cAMP-induced Cytoskeleton Rearrangement Increases Calcium Transients through the Enhancement of Capacitative Calcium Entry*

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In this study, we investigated the possibility that changes in cell morphology might result in changes in intracellular calcium signaling. A common means of inducing morphological changes in type I astrocytes has been prolonged application of long-lasting cAMP analogues. Astrocytes, which differentiate after prolonged exposure to cAMP analogues, changed from a flat polygonal form to a stellate process-bearing appearance (1). Type I astrocytes differentiated with a long-term exposure to cAMP showed changes in biochemical properties such as an increase in the production of inositol (1,4,5)-trisphosphate (InsP3) in response to both α1-adrenergic agonists (2) and bradykinin (BK) (3). In addition, long-term cAMP-induced differentiation modifies membrane ionic conductance (4, 5).

Cultured type I astrocytes express a wide array of second messenger-coupled receptors (for review see Ref. 6). Among these receptors, F2Y, a subclass of purinergic receptors, activated by ATP (7) and BK receptors (3, 8) are coupled to phospholipase C (PLC). Their activation causes calcium mobilization from intracellular calcium stores via PLC-induced InsP3 production (for review see Ref. 9).

Store emptying generates a putative signal (10) that induces the opening of the store-operated calcium channel (SOC) at the level of the cell membrane, also known as the calcium release-activated calcium channel (CRAC) (for review see Ref. 11), which allows calcium into the cells from the extracellular space. SOC/CRAC channels may be coupled to intracellular calcium stores through a physical connection (9, 12–15). Alternatively, the stores can refill by promptly capturing cytosolic calcium, admitted in the cells after the opening of the SOC/CRAC (16). Calcium entry through the SOC/CRAC, activated by the depletion of the intracellular calcium stores, is also known as capacitative calcium entry (CCE).

Disorganization of the cell shape induced by disrupting cytoskeleton organization was previously found to differentially affect calcium transients and CCE in different cell types (17, 18). In this study, we provide evidence that active and rapid rearrangement of astrocyte morphology induced by activation of the protein kinase A (PKA), is responsible for enhancement of InsP3-induced cytosolic calcium concentration ([Ca2+]i) elevation via an enhanced CCE. Enhanced CCE, in turn, is associated with reorganization of the spatial relationship between the outer cell membrane and the endoplasmic reticulum (ER).

EXPERIMENTAL PROCEDURES

Preparation of Primary Cultures of Rat Cortical Type I Astrocytes—Embryonic type I astrocyte cultures were obtained from E-17 fetuses

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(19). Briefly, dissected cortex from 17-day-old fetuses, cut into small fragments, were exposed to Papain (Worthington) and mechanically dissociated. The cell suspension was centrifuged and plated onto non-coated 25-cm² flasks in Dulbecco's modified Eagle's medium (containing 10% fetal bovine serum (HyClone, Logan, UT) (10⁶ cells/flask)(Corn-ing-Costar, NJ). After 6–8 h, unattached cells, mainly neurons, were washed away. This protocol yielded at least 95% pure type I astrocytes as characterized by glial fibrillary acid protein (GFAP).

Post-natal type I astrocytes were prepared as described previously (20). Dissected cortex from 2-day-old Wistar rats were digested with trypsin (ICN Biomedical Inc., Costa Mesa, CA) (0.125% in calcium-free PBS), and then mechanically dissociated to single cells.

**Differentiation, Pretreatments, and Experimental Protocols**—Astrocytes were exposed to 1 mM Bt₂cAMP for 30 min before Fura-2 loading. Successively, the cells were loaded with Fura-2 always in the presence of pCa 6.3 conventional fluorescence microscope (Zeiss, Germany) equipped with a 40× objective. The preparations were observed with a coverslips were mounted on a glass slide with Vectashield (Vector Laboratories, Burlingame, CA). The preparations were observed with a conventional fluorescence microscope (Zeiss, Germany) equipped with a 63× magnification oil immersion objective (Zeiss).

**Indirect Immunofluorescence**—Astrocytes plated on glass coverslips were washed twice in PBS and fixed in 4% paraformaldehyde in PBS. After washing in PBS containing glycine, the cells were permeabilized (0.1% Triton X-100). Nonspecific sites were saturated with 0.2% bovine serum albumin. The preparations were exposed to anti-GFAP monoclonal antibody 1:200 (anti-mouse IgG conjugated with rhodamine). After two washes in PBS-bovine serum albumin, the preparations were exposed for 20 min to the secondary antibody 1:200 (anti-mouse IgG conjugated with rhodamine). After two washes in PBS-bovine serum albumin the coverslips were mounted on a glass slide with Vectashield (Vector Laboratories, Burlingame, CA). The preparations were observed with a conventional fluorescence microscope (Zeiss, Germany) equipped with a 63× magnification oil immersion objective (Zeiss).

**ER Labeling and Living Cell Confocal Microscopy**—Astrocytes were incubated for 15 min with 1 µM ER-Tracker (Molecular Probes, Eugene, OR), washed for an additional 15 min, and then kept in saline solution until observed. When ready, the coverslips were mounted in saline solution on a glass slide and observed with a Zeiss confocal microscope. We acquired a differential interference contrast image and a series of fluorescence images at 0.5 µm intervals in the z axis using 364 nm excitation and collecting with a 395 nm long pass emission filter. A 63× lens, coupled to a software zooming capability, was used to produce single cell images.

**InsP₃ Assays**—InsP₃ accumulation was performed as described previously (19). Near-confluent astrocytes were switched to serum-free, myo-[2-3H]inositol (30 Ci/mmol) (American Radiolabeled Chemicals, Inc., St. Louis, MO), and labeled for 36 h. After labeling, cells were washed in KRB containing in mM: NaCl, 125; KCl, 5; MgSO₄, 1; CaCl₂, 1; glucose, 5.5; HEPES, 20 mM pH was set at 7.2.

**FIG. 1. Pharmacological manipulation of type I astrocytes morphology.** Astrocytes were treated for 100 min with 1 mM Bt₂cAMP in the absence or presence of 300 nM KT-5720, 10 µM CytoD or nocodazole, respectively. Next, the cells were fixed and immunostained for GFAP (a–e) and observed with an inverted epifluorescence microscope equipped with a 63× lens. Panel a displays GFAP immunoreactivity in a control astrocyte culture. Panel b shows GFAP immunoreactivity in Bt₂cAMP-treated astrocytes. Panels c, d, and e illustrate the effect of 300 nM KT-5720, 10 µM CytoD, and 10 µM nocodazole, respectively, in the presence of 1 mM Bt₂cAMP.

**FIG. 2. Effects of the differentiation on agonist-induced calcium transients.** [Ca²⁺], in single cells, expressed as average of the entire cell area, were measured. Thereafter the values derived from all the cells were averaged and graphed as average ± S.E. Panel a shows the effect of ATP-induced [Ca²⁺], elevation in undifferentiated (open circles) and differentiated (closed circles) astrocytes. The inset to panel a displays the statistical analysis of the experimental data at the maximal value of the spike. In panel b the response to BK in undifferentiated (open triangles) and differentiated (closed triangles) astrocytes is shown. Inset I to panel b displays the statistical analysis of the experimental data at the maximal value of the spike. The first arrowhead indicates the time at which ATP or BK was added. The second arrowhead indicates the washout of the agonist. *p ≤ 0.05, versus value in undifferentiated cells.
100–200 mesh, mixed 1:3 in water) (Bio-Rad). Water was used to elute the unbound. Total InP$_1$, InP$_2$, and InP$_3$ were individually recovered from the resin with a serial elution with increasing concentration of AF in 0.1 M formic acid (0.4 M AF for InP$_1$, 0.8 M AF for InP$_2$, 1.2 M AF for InP$_3$). Finally, collected samples were mixed with appropriate volumes of scintillation mixture and counted for 4 min.

**Single Cell [Ca$^{2+}$], Measurements—**Nearly confluent astrocyte cultures seeded on glass coverslips (Assistent, Germany) were washed once in KRB. After washing, cells were loaded with 2 μM Fura-2/AM (Molecular Probes) for 22 min at room temperature to minimize dye compartmentalization in subcellular compartment, under continuous gentle agitation. After loading, the cells were washed once with fresh KRB and then kept for an additional 22 min in Fura-2/AM-free KRB (19). Ratio measurements were acquired every 2 s. The experimental data were analyzed with the software MetaFluor (Universal Imaging, West Chester, PA). Briefly, an area corresponding to the entire cell surface was delimited by using the editing capability of the software. The experimental ratio values, derived from the entire cytosolic area, obtained by delimiting the profile of the cell and averaging the signals within the delimited area, were converted in cytosolic calcium concentration using a titration calibration curve obtained in living cells exposed to known extracellular calcium concentration in the presence of 5 μM ionomycin (containing 5.2% calcium). The following are the ratio values measured in astrocytes: 760 nM Ca$_{ext}$ R$_{340/380}$ = 18; 1,260 μM Ca$_{ext}$ R$_{340/380}$ = 32. R$_{340/380}$ at 0 Ca$_{ext}$ = 0.29; R$_{340/380}$ at 10 mM Ca$_{ext}$ = 60.

**Materials—**All substances were from Sigma unless stated otherwise.

**Statistical Analyses—**Experiments were performed at least three times on different cell preparations. The plots were created by using the editing capability of the software. Statistical significance was determined by analysis of variance followed by t test. Differences were considered significant for p ≤ 0.05.

**RESULTS**

**Differentiation of Rat Cortical Type I Astrocytes with a Brief Treatment of Bt$_2$cAMP—**Flat polygonal type I astrocytes (Fig. 1a) exposed to 1 mM Bt$_2$cAMP (Fig. 1b) or 100 μM forskolin (not shown) for 100 min, exhibited a process-bearing cell phenotype, as shown by imaging of GFAP immunoreactivity.

**Agonist-induced Calcium Responses in Undifferentiated and Differentiated Astrocytes—**ATP- and BK-induced statistically significant responses in astrocytes (Fig. 2a and b), whereas about 75% of the astrocytes responded to BK (Fig. 2, panel b, inset II) (9). In differentiated astrocytes, we observed a potentiation of ATP responses at all concentrations tested (Fig. 2, panel a, open circles versus closed circles). Both the initial, “spike-like,” phase and the sustained phase of intracellular calcium elevation were increased in differentiated cells, as compared with undifferentiated cells (Fig. 3, panels a and b, closed circles versus open circles). The spatial development of ATP response was also analyzed. In control astrocytes, the responses began in the periphery of the cell body and extended into the perinuclear area (Fig. 4, panels a–d). The decay of the responses began from the perinuclear area and subsequently extended into the periphery of the cell. The entire response was very fast (8 s in toto). In differentiated astrocytes [Ca$^{2+}$], increased initially in the astrocyte prolongment and in the periphery of the cells and extended to the soma (panels e–n). Astrocyte processes and the regions immediately adjacent to the inner cell membrane showed a more marked [Ca$^{2+}$] elevation. In addition, differentiation significantly prolonged the duration of the [Ca$^{2+}$] elevation. In fact, as shown in Fig. 4, the response lasted 4 frames (panels a–d) in control, and 8 frames (panels e–n) in differentiated astrocytes (given the 2 s interval between each frame acquisition, the response lasted 8 s in controls and 16 s in differentiated astrocytes (Fig. 4)).

Statistically significant BK responses (Fig. 2b, inset I) were recorded in 70–80% of the cells studied as already described by other authors (Fig. 2b, inset II) (9). The percentage of cells responding to BK was not affected by differentiation (Fig. 2b, inset II). ATP responses were also increased when differentiation of astrocytes was achieved by exposure to 100 μM forskolin for 100 min ([Ca$^{2+}$], peak in undifferentiated astrocytes was 620 ± 35 nM versus 1,650 ± 120 μM in forskolin differentiated astrocytes, n = 76, p ≤ 0.05). A few minutes exposure to Bt$_2$cAMP, which was unable to induce morphological changes, did not increase ATP responses ([Ca$^{2+}$], 580 ± 50 nM, n = 46). In the absence of extracellular calcium, however, both undifferentiated and differentiated astrocytes displayed similar [Ca$^{2+}$] elevation in response to ATP ([Ca$^{2+}$], in undifferentiated astrocytes in absence of extracellular calcium and 100 μM EGTA = 420 ± 70, n = 65; [Ca$^{2+}$], in differentiated cells in absence of extracellular calcium and 100 μM EGTA = 480 ± 100 nM, n = 66). Blockade of PKA with 300 nM KT-5720 (for review see Refs. 20 and 21) (a gift from Kamiya Biomedical Co., Seattle, WA), although did not decrease calcium transients evoked by ATP (Fig. 5), prevented the morphological changes induced by cAMP (Fig. 1c) and the enhancement of the [Ca$^{2+}$] elevation in response to ATP (Fig. 5).
Fig. 4. Space- and time-resolved imaging of ATP response. Fura-2 fluorescence at 340 and 380 was imaged using a 40× lens every 2 s, ratioed and converted into [Ca²⁺], [Ca³⁺], was expressed as pseudocolor ranging from blue to red as shown by the color bar in the top right corner of the figure. The arrowheads in panels a and e indicate the direction of perfusion flow. Panels a–d show the responses to 10 µM ATP in undifferentiated astrocytes. ATP reached the cells at the image a. The successive ratio images display the development of the [Ca²⁺] elevation. Panels e–h show responses to 10 µM ATP in differentiated astrocytes. ATP reached the cells in image e. The arrows in panel f indicate the early and high increase of [Ca²⁺], in the processes (orange) and in the periphery (white) of the cell, respectively. The white arrow in panel h indicates the initial increase of [Ca²⁺], in the cell soma. Panels i and j display traces of the response to ATP in two representative experiments in control and differentiated astrocytes, respectively. Each line represents the trace derived from the entire cell imaged.

cytes—ATP-induced InsP₁ accumulation was similar in both undifferentiated and differentiated cells (Fig. 6a). However, in differentiated astrocytes the stimulation reached a plateau at a lower concentration of ATP than in undifferentiated cells. Although there were no significant changes in the accumulation of InsP₁, we also tested whether the production of InsP₃ was changed in the two cell types. InsP₃ production after a 15 s stimulation with ATP was unaffected by differentiation (Fig. 6, inset to panel a). Because it has been reported that long term treatment with cAMP analogues can affect both BK- and α₁-adrenergic-induced InsP₁ production in post-natal astrocytes (2, 3), we also studied InsP₁ production in post-natal astrocytes (Fig. 6b). In post-natal astrocytes, the treatment with Bt₂cAMP for 100 min caused cell differentiation (not shown) and potentiated ATP-induced [Ca²⁺], elevation ([Ca²⁺], after ATP stimulation in undifferentiated astrocytes = 654 ± 22 nM, n = 58; [Ca²⁺], after ATP stimulation in differentiated astrocytes = 1,753 ± 500 nM, n = 77; p < 0.05). As was observed in embryonic astrocytes, InsP₁ generation was not affected by differentiation in post-natal astrocytes either (Fig. 6b).

Effect of Differentiation on Calcium Release from Intracellular Stores—In the absence of extracellular calcium, the release of calcium from intracellular stores induced by 20 µM thapsigargin (an irreversible inhibitor of the smooth endoplasmic reticulum Ca-ATPase; for review see Ref. 22) was identical in both undifferentiated and differentiated astrocytes (Fig. 7a). However, in the presence of extracellular calcium, the increase of [Ca²⁺], caused by 20 µM thapsigargin was significantly larger (see Fig. 7c) in differentiated astrocytes than in undifferentiated cells (Fig. 7b).

Effect of Actin Stress Fiber Depolymerization on the Potentiation of ATP- and Thapsigargin-induced [Ca²⁺]. Elevation—Treatment of astrocytes with 10 µM cytochalasin D (CytD) did not significantly affect morphology (not shown) and slightly increased ATP responses in undifferentiated cells (Fig. 8a). Treatment with 10 µM CytD, however, prevented the morphological changes induced by Bt₂cAMP (Fig. 1a), and the potentiation of ATP-induced [Ca²⁺], elevation (Fig. 8a). Thapsigargin-induced [Ca²⁺], elevation in undifferentiated cells was decreased by 10 µM CytD (Fig. 8b). In differentiated cells the potentiation of thapsigargin-induced [Ca²⁺], elevation was abolished by actin depolymerization (Fig. 8b).

Effect of the Anti-tubulin Drug Nocodazole on the Potentiation of ATP- and Thapsigargin-induced [Ca²⁺]. Elevation—Treatment of astrocytes with the anti-tubulin drug nocodazole, at 10 µM, prevented cAMP-induced morphological differentia-
PKA-induced Morphology Change Increases Ca^{2+} Transients

FIG. 5. Effect of PKA blockade on ATP response. The inhibition of PKA activity by KT-5720 blocked the increase of ATP-induced [Ca^{2+}], elevation in cAMP-treated astrocytes. The response to ATP was measured in undifferentiated astrocytes (open bar), in undifferentiated astrocytes in the presence of 300 nM of KT5720 (hatched bar), in differentiated astrocytes (solid bar), and in B_t cAMP-treated astrocytes pre-exposed to 300 nM KT5720 (square-filled bar). Thereafter, the peak value of [Ca^{2+}], from individual treatments was averaged and expressed as a bar graph and used to perform statistical analysis. * p ≤ 0.05 versus value in control cells; ** p ≤ 0.05 versus value in differentiated cells.

FIG. 6. Effect of differentiation on InsP_1 and InsP_3 production induced by ATP. We studied InsP_3 production in both embryonic and post-natal astrocytes. Panel a displays the concentration-response curve of InsP_3 generation in response to ATP in undifferentiated (open circle) and differentiated (closed circle) cells in embryonic astrocytes. The inset to panel a displays InsP_3 production in basal conditions (open bars) and after 15 s of stimulation with 10 μM ATP (solid bars) in undifferentiated and differentiated cells, respectively, in embryonic astrocytes. Panel b displays the accumulation of InsP_3 in basal conditions (open bars) and after stimulation with 10 μM ATP (solid bars) in undifferentiated and differentiated cells, respectively, in astrocytes prepared from a 2-day-old rat.

distribution of type I astrocytes (Fig. 1e). Nocodazole treatment increased ATP-induced [Ca^{2+}], elevation in control cells (Fig. 9a). However, B_t cAMP-treated astrocytes exposed to nocodazole showed a reduction of the calcium transient evoked by ATP as compared with differentiated astrocytes not treated with nocodazole (Fig. 9a). In control cells, thapsigargin-induced calcium mobilization was not affected by nocodazole (Fig. 9b). In differentiated cells the treatment with nocodazole eliminated the potentiation of [Ca^{2+}], elevation observed after exposure to thapsigargin (Fig. 9b).

Distribution of the Endoplasmic Reticulum in Polygonal and Differentiated Type I Astrocytes—In flat polygonal type I astrocytes, the internal membrane-delimited compartments are well visible in the periphery of the cell, whereas in the perinuclear space ER structures are grouped with other intracellular membrane delimited compartments such as the Golgi apparatus (Fig. 10, a and b). Isolated and rare single tubular structures, reaching out of the cell membrane border, were present in the periphery of the cell (arrowheads, Fig. 10, a and b). Fully differentiated astrocytes were smaller, thicker, and rounder and had extended large processes (Fig. 10c). In this reconfigured morphology, the cells showed a marked condensation of the internal membrane-delimited compartments all across the cell (Fig. 10d).

DISCUSSION

Capacitative calcium entry is a mechanism whereby intracellular calcium stores are refilled (9, 12–15, 16). The maintenance of the filled state of intracellular stores is involved in cell survival as the prolonged depletion of calcium reservoirs causes cell death and apoptosis (23).

Our data indicated that the fast occurring morphological rearrangement of type I astrocytes from a flat polygonal appearance to a stellate process-bearing one, induced by exposure to B_t cAMP or forskolin, was associated with larger and longer calcium transients in response to neurotransmitters activating PLC, particularly within the newly created processes and in regions immediately adjacent to the cell membrane. Potenti-
InsP$_3$ accumulation and InsP$_{1,3}$ production were both unaffected by differentiation. Although PLC activity was not changed by differentiation, differences in InsP$_{1,3}$-receptor sensitivity, and direct modulation of the InsP$_{1,3}$-activated calcium channel on the ER could have played a role in the enhancement of calcium transients in differentiated cells. However, the fact that thapsigargin-induced calcium transients, which do not rely upon InsP$_3$ receptor activation, were also enhanced by astrocyte differentiation, supports the involvement of a mechanism downstream to InsP$_3$ receptor activation. To further characterize the mechanism underlying enhancement of the calcium transients in differentiated cells, we analyzed the contribution of extracellular calcium to the potentiation of calcium transients in differentiated astrocytes. In the absence of extracellular calcium to the potentiation of calcium transients in differentiated cells, we analyzed the contribution of calcium stored in other intracellular compartments, and the amount of calcium stored in other intracellular calcium reservoirs (that were not sensitive to InsP$_3$ but could have been released by calcium itself) were not involved in the enhancement of the calcium transients in differentiated cells. On the other hand, the absolute dependence on extracellular calcium for the enhancement of calcium transients, observed in differentiated astrocytes, indicates that an increase of calcium entry was clearly involved in the potentiation of calcium responses in differentiated cells. Thus, our data suggests that calcium entrance from the extracellular space was increased in Bt$_2$cAMP-treated astrocytes, only if they had undergone shape changes.

Cell morphology has been shown to play a role in agonist-induced calcium mobilization in fibroblasts (17), in thapsigargin-induced store depletion, and in the associated CCE in endothelial cells (18). Because CAMP-induced differentiation causes the rearrangement of the actin-formed stress fiber (1), we induced actin to depolymerize with CytD before exposing the cells to Bt$_2$cAMP. As expected, in the presence of CytD, the cells did not reshape and maintained their flat polygonal appearance, although PKA was being activated by CAMP. The prevention of the shape changes in Bt$_2$cAMP-treated cells, by CytD, avoided the potentiation of ATP- and thapsigargin-induced [Ca$^{2+}$]$_i$ elevation. Both PKA activity in the cell cytosol and PKA localization at level of the cell membrane, where CRAC channels are localized, have been reported not to be affected by CytD pretreatment (24–26). Therefore, it seems likely that morphological changes involving the rearrangement
of stress fibers play a critical role in the potentiation of the CCE. CytD was reported to decrease CCE in endothelial cells without affecting agonist-induced calcium transients (18). However, it was also reported that CytD was able to induce shape changes in fibroblasts that were associated with the loss of the sensitivity to ATP in the presence of preserved thapsigargin and CCE responses (17). We did observe both a slight potentiation of ATP response and a more pronounced inhibition of thapsigargin-induced calcium mobilization, following CytD treatment in undifferentiated cells, the morphology of which did not undergo appreciable changes after CytD pretreatment (not shown), although actin filaments were disassembled by the drug (not shown). This suggests that, in type I astrocytes, disrupting stress fibers with CytD is not sufficient to abolish calcium transients, unless a dramatic change of the shape of the cells is achieved. However, the specific inhibition of the stress fiber reorganization was able to block the cAMP-induced differentiation and the associated potentiation of the calcium transients.

To confirm this interpretation, we tested the effect of nocodazole, an agent that impairs tubulin cytoskeleton organization. Nocodazole pre-exposure blocked the differentiation of the astrocytes obtained with cAMP. Noticeably, nocodazole itself caused a consistent increase of the calcium transients elicited by ATP in control astrocytes, although it did not affect thapsigargin-induced calcium mobilization. This unexpected and selective increase in ATP response by nocodazole, without affecting thapsigargin action, may be due to an interference of the drug with the ATP system, unrelated to its activity as a tubulin depolymerizer. However, nocodazole-treated cells in the absence or in the presence of Bt$_2$cAMP showed similar responses, suggesting that although the response to ATP was increased by nocodazole itself, nocodazole treatment was able to prevent calcium signaling changes due to shape changes. In addition, the ability of nocodazole to block the potentiation of thapsigargin-induced calcium mobilization, without affecting thapsigargin effect, in cAMP-treated astrocytes showed clearly the dependence of the calcium transient enhancement on the acquisition of the new morphology.

Two hypotheses have been proposed to explain how calcium flowing through the SOC/CRAC during CCE can be captured and stored in the ER compartments and thus contribute to the spike or the plateau phase of the single calcium transient. Calcium, according to the preferential pathway hypotheses, could be directly sequestered within intracellular stores because of a physical association between the SOC/CRAC and the SERCA-ER complex (9, 12–15). Alternatively, calcium ions, once admitted into the cytosol by SOC/CRAC opening, might be quickly removed from the cytosol and accumulated in the ER by the SERCA (diffuse pathway) (16). Regardless of which of the two mechanisms is operating in type I astrocytes, it is likely that the reorganization of the spatial relationship between ER

**FIG. 9.** Effect of the anti-tubulin drug nocodazole on ATP- and thapsigargin-induced $[\text{Ca}^{2+}]_{i}$ elevation. $[\text{Ca}^{2+}]_{i}$ values obtained at the peak of the response were averaged and graphed as bars ± S.E. In panel a, the effect of anti-tubulin drug nocodazole on ATP-induced intracellular calcium mobilization in undifferentiated and differentiated astrocytes is displayed. Nocodazole-treated astrocytes (hatched bar) showed a higher response to ATP than untreated undifferentiated astrocytes (open bar). However, nocodazole pretreatment (square-filled bar) reversed the potentiation of ATP response in differentiated astrocytes (solid bar) to the values observed in the nocodazole-treated undifferentiated cells. In panel b, the effect of nocodazole on thapsigargin-induced intracellular calcium mobilization in undifferentiated and differentiated astrocytes is displayed. Nocodazole-treated undifferentiated astrocytes (hatched bar) showed a similar response to thapsigargin as undifferentiated cells (open bar). Nocodazole pretreatment (square-filled bar) completely reversed the potentiation of thapsigargin response in differentiated astrocytes (solid bar). * $p \leq 0.05$ versus value in undifferentiated cells; ** $p \leq 0.05$ versus value in differentiated cells.

**FIG. 10.** Analysis of the ER distribution and association with cell membrane in control and differentiated astrocytes. ER was labeled in living cells by means of the ER tracker and analyzed with a confocal microscope, equipped with a 63× lens for ER-associated fluorescence. Images were then software zoomed to resolve single astrocytes. Undifferentiated astrocytes (panel a) present a higher density of ER membranes in the perinuclear area, whereas the density of the tubular network is decreased in the peripheral part of the cells (panel b). The arrowheads indicate some connection between the tubular ER structures and the cell membrane. Differentiated astrocytes (panel c) showed a more condensed ER organization (panel d) without appreciable areas free of fluorescence signal across the cell.
structures and cell membrane may cause a change in the efficiency of calcium mobilization as results of the improved refilling of the intracellular calcium stores. This would, in turn, result in larger calcium transients. We assessed the arrangement of the ER in undifferentiated and differentiated cells. In undifferentiated astrocytes, ER structures were largely evident in the periphery of the cells where a tubular network of membrane delimited structures were identifiable. In addition, grouped in the perinuclear region of the astrocytes, ER structures and the Golgi complex were identified. In differentiated cells, at least as a consequence of the marked reorganization of the cell shape, the ER was so condensed that areas of the cytoplasm free of ER structures were virtually absent. Such a marked rearrangement may, in turn, increase the availability of calcium flowing from the extracellular space to provide for refilling of the stores during CCE, through a closer association of the outer cell membrane and ER structures.

In conclusion, our data indicates that an active and rapid remodeling of type I astrocyte morphology from an expanded epithelial-like to a condensed process-bearing shape is induced by the activation of the cAMP-PKA system. This remodeling appears to cause a cytoskeleton-driven spatial reorganization of the relationship between the plasma membrane and ER structures, thereby increasing calcium flow into the cells during CCE, that, in turn, enhances agonist-induced $[Ca^{2+}]_i$ elevation.

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