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Rilmenidine Elevates Cytosolic Free Calcium Concentration in Suspended Cerebral Astrocytes

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Abstract: Rilmenidine, a ligand for imidazoline and α_2-adrenergic receptors, is neuroprotective following focal cerebral ischemia. We investigated the effects of rilmenidine on cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in rat astrocytes. Rilmenidine caused concentration-dependent elevation of $[Ca^{2+}]_i$, consisting of a transient increase (1–100 μM rilmenidine) or a transient increase followed by sustained elevation above basal levels (1–10 mM rilmenidine). A similar elevation in $[Ca^{2+}]_i$ was induced by the imidazoline ligand cirazoline. The transient response to rilmenidine was observed in Ca^{2+}-free medium, indicating that rilmenidine evokes release of Ca^{2+} from intracellular stores. However, the sustained elevation of Ca^{2+} was completely dependent on extracellular Ca^{2+}, consistent with rilmenidine activating Ca^{2+} influx. Pretreatment with thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, abolished the response to rilmenidine, confirming the involvement of intracellular stores and suggesting that rilmenidine and thapsigargin activate a common Ca^{2+} influx pathway. The α_2-adrenergic antagonist rauwolscine attenuated the increase in $[Ca^{2+}]_i$ induced by clonidine (a selective α_2 agonist), but not the response to rilmenidine. These results indicate that rilmenidine stimulates both Ca^{2+} release from intracellular stores and Ca^{2+} influx by a mechanism independent of α_2-adrenergic receptors. In vivo, rilmenidine may enhance uptake of Ca^{2+} from the extracellular fluid by astrocytes, a process that may contribute to the neuroprotective effects of this agent. **Key Words:** Astrocytes—Rilmenidine—Calcium—Imidazoline receptors—Clonidine.
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Rilmenidine, clonidine, and related compounds bind to both imidazoline receptors and α_2-adrenoceptors. Imidazoline receptors are distinguished from α_2-adrenoceptors on the basis of anatomical distribution (Coupry et al., 1989; Kamisaki et al., 1990), signal transduction mechanisms (Michel et al., 1990), binding profiles (Ernsberger et al., 1987, 1988), and insensitivity to catecholamines and GTP analogues (Wikberg and Uhlén, 1990; Bricca et al., 1993, 1994). Cell lines transfected with α_2-adrenoceptor genes do not demonstrate imidazoline receptor binding (Kamisaki et al., 1990). Imidazoline receptors are divided into subtypes I_1 and I_2. The I_1 subtype [labeled by [^{3}H]clonidine (Ernsberger et al., 1995)] is present on neuronal plasma membranes (Ernsberger et al., 1995) within the rostral ventrolateral medulla, hippocampus, hypothalamus, and striatum (Kamisaki et al., 1990), and may be linked to G proteins (Bricca et al., 1994; Ernsberger et al., 1995). In contrast, the I_2 subtype [labeled by [^{3}H]idazoxan (Regunathan et al., 1993)] is localized to astrocytic mitochondrial membranes (Tesson and Parini, 1991) and is not linked to G proteins (Regunathan et al., 1991).

It has been shown recently that certain imidazoline receptor ligands are neuroprotective following an ischemic insult. The first indication that imidazoline receptors are involved in neuroprotection was from Gustafson et al. (1989, 1990), who showed that idazoxan, an imidazoline receptor ligand that is also an α_2-adrenergic receptor antagonist, reduces the infarct size in rats following occlusion of the middle cerebral artery. It was later demonstrated that both idazoxan and rilmenidine reduce the infarct size by a mechanism distinct from both α_2-adrenoceptor interaction and local cerebral blood flow elevation (Maiese et al., 1992). In addition, the neuroprotective effect of rilmenidine has been shown to be dose-dependent (Maiese et al., 1992).

Within the cerebral cortex, neuronal imidazoline receptors are absent (Kamisaki et al., 1990), whereas cortical astrocytes express the I_2 subtype on the outer mitochondrial membrane (Wikberg and Uhlén, 1990; Tesson and Parini, 1991; Mallard et al., 1992). Therefore, it is likely that rilmenidine induces neuroprotection in cortex via activation of astrocytic imidazoline receptors. Although the signal transduction pathway of imidazoline receptors remains unclear, it is possible

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Abbreviations used: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; I_1, I_2, and I_3, subtype 1, 2, and 3 imidazoline receptor, respectively.

that rilmenidine stimulates astrocytes to buffer potentially neurotoxic levels of extracellular Ca^{2+}. In this regard, rilmenidine has been shown to induce uptake of $^{45}Ca^{2+}$ in a concentration-dependent manner in minislices of cerebral cortex (Regunathan et al., 1995).

In this study, we have investigated the action of rilmenidine on the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in cerebral astrocytes. In addition, we have examined whether the effects of rilmenidine are dependent on α_2-adrenoceptor activation.

EXPERIMENTAL PROCEDURES

Materials

Minimum essential medium containing 1.8 m*M* $CaCl_2$ was prepared according to the method outlined by Dixon and Wilson (1995). Horse serum and Hanks' balanced salt solution without $CaCl_2$ were obtained from GIBCO Laboratories (Burlington, ON, Canada). Pluronic F-127 and both the acetoxymethyl ester and pentapotassium salt of indo-1 were purchased from Molecular Probes (Eugene, OR, U.S.A.). ATP, EGTA, trypsin, phentolamine, clonidine, and thapsigargin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Rauwolscine was acquired from Research Biochemicals International (Natick, MA, U.S.A.). Cirazoline was obtained from Tocris (Ballwin, MO, U.S.A.). Rilmenidine was a gift from Servier Laboratories (Paris, France). Falcon brand culture dishes were purchased from VWR Canada (Mississauga, ON, Canada). Trypsin solution consisted of nominally Ca^{2+}- and Mg^{2+}-free buffer containing trypsin (0.05%) and EDTA (0.5 m*M*). Na^+ buffer contained 135 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 10 m*M* glucose, and 20 m*M* HEPES, pH 7.30 ± 0.02, osmolarity 290 ± 5 mosmol/L. Ca^{2+}-free Na^+ buffer had additional NaCl substituted for $CaCl_2$.

Cells and cell culture

Primary cultures of type 1 astrocytes were prepared from 1-day-old Wistar rats according to the method of Dixon and Wilson (1995). Astrocytic cultures were maintained for 14–18 days before being used for experiments. Immunohistochemical analysis indicated that 95% of the cultured cells expressed the astrocytic marker, glial fibrillary acidic protein (data not shown).

Measurement of $[Ca^{2+}]_i$

Primary cultures of astrocytes were incubated with Hanks' balanced salt solution containing Pluronic F-127 (0.03%) and indo-1 acetoxymethyl ester (5 μM) at 37°C for 10 min. The cells were then washed with 2 ml of Na^+ buffer and subsequently harvested by exposure to 3 ml of trypsin solution (37°C) per 60-mm dish for 1 min. Trypsinization was halted by the addition of minimum essential medium supplemented with horse serum (10%). Cells were sedimented at 225 *g* for 5 min and resuspended in 2 ml of Na^+ buffer at $\sim 1 \times 10^6$ cells/ml. Where indicated, experiments were performed using nominally Ca^{2+}-free Na^+ buffer supplemented with EGTA (1 m*M*). The cell suspension was placed in a fluorimetric cuvette and maintained at 37°C with constant stirring. $[Ca^{2+}]_i$ was monitored using a fluorimeter (model RF-M2004, Photon Technology International, London, ON, Canada) with an excitation wavelength of 355 nm and emission wavelengths of 405 and 485 nm. The software for the system (Oscar, PTI) calculated $[Ca^{2+}]_i$ from the equation $[Ca^{2+}]_i = K_D\,[(R - R_{min})/(R_{max} - R)]\beta$, where K_D is the dissociation constant for the indo-1–Ca^{2+} complex having a value of 250 n*M*, R is the fluorescence intensity at 405 nm divided by the intensity at 485 nm, R_{max} and R_{min} are the fluorescence ratio values under saturation and low Ca^{2+} conditions, respectively, and β is the ratio of fluorescence values for low and saturating Ca^{2+} concentrations measured at 485 nm. To obtain saturating $[Ca^{2+}]_i$, the fluorescence of indo-1 pentapotassium salt in Na^+ buffer (1 m*M* Ca^{2+}) was measured. Low $[Ca^{2+}]_i$ was obtained by using Ca^{2+}-free Na^+ buffer containing EGTA (10 m*M*) and alkalinizing the buffer with Tris.

Data analysis

The change in $[Ca^{2+}]_i$ was calculated by measuring either the peak height of the response above basal level or the average height during the first 100 s of the response. The latter means of measurement was accomplished using Mocha image analysis software (Jandel Scientific) to determine the area between the response curve and the predicted basal level for the first 100 s following injection of drug. All experiments were done on three or more astrocyte preparations from individual litters of rats (except for cirazoline experiments, in which two litters were used). The n values (stated in parentheses) represent measures from astrocyte cultures grown in separate dishes. Data are presented as means ± SEM. Comparisons between mean values based on a single level of treatment were evaluated using the paired *t* test (two-tailed). A p value of <0.05 was considered significant. No significant changes in $[Ca^{2+}]_i$ were caused by the administration of the solvents for all substances tested.

RESULTS

$[Ca^{2+}]_i$ response to rilmenidine

To examine the effect of rilmenidine on $[Ca^{2+}]_i$ in astrocytes, 0.1 m*M* (final concentration) was added to indo-1-loaded astrocytes suspended in Na^+ buffer. Rilmenidine consistently produced significant increases in $[Ca^{2+}]_i$ immediately following its addition to the buffer (Fig. 1A). For comparison, we also illustrate the increase in $[Ca^{2+}]_i$ induced by 0.1 m*M* ATP, which activates astrocytic purinergic receptors, resulting in the release of Ca^{2+} from intracellular stores and Ca^{2+} influx (Peuchen et al., 1996). Rilmenidine and ATP induced an increase in the fluorescence intensity at 405 nm concurrently with a decrease in fluorescence intensity at 485 nm, indicating a true elevation in $[Ca^{2+}]_i$ (Fig. 1B). Rilmenidine was dissolved in water. An equivalent amount of water (20 μl) did not elicit an increase in $[Ca^{2+}]_i$, whereas 0.001–0.1 m*M* rilmenidine evoked a transient elevation in $[Ca^{2+}]_i$ that returned to basal levels usually within 50 s (Fig. 2A). Higher concentrations of rilmenidine (1–10 m*M*) induced an increase in $[Ca^{2+}]_i$ followed by a sustained elevation that did not return to basal levels within 200 s. Representative concentrations of 0.1 and 10 m*M* rilmenidine were chosen for the remainder of the experiments to characterize the mechanisms underlying Ca^{2+} increases induced by rilmenidine.

Basal levels of $[Ca^{2+}]_i$ in these experiments were between 120 and 340 n*M*. The correlation between

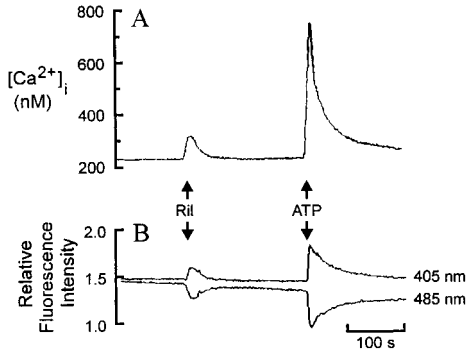

FIG. 1. A: Examples of the increase in $[Ca^{2+}]_i$ in suspended astrocytes evoked by rilmenidine (Ril; 0.1 m*M*) and ATP (0.1 m*M*). $[Ca^{2+}]_i$ was monitored in indo-1-loaded cerebral astrocytes by fluorescence spectrophotometry. **B:** Traces are responses to rilmenidine and ATP at emission wavelengths of 405 and 485 nm. The fluorescence emission from the Ca^{2+}-bound form of the dye predominates at 405 nm, whereas emission from the unbound dye predominates at 485 nm. Both rilmenidine and ATP induced an increase in fluorescence at 405 nm and a simultaneous decrease in fluorescence intensity at 485 nm, indicating that the changes in fluorescence reflect actual changes in $[Ca^{2+}]_i$.

basal values and the magnitude of the peak height above basal level for each concentration of rilmenidine was examined using GraphPad Instat software. There was no significant correlation between the magnitude of the elevations in $[Ca^{2+}]_i$ evoked by all rilmenidine concentrations and the initial basal value of $[Ca^{2+}]_i$.

Concentration-dependence curves were generated by two approaches. First, peak height above basal level for each rilmenidine concentration was calculated. Second, the average increase above basal level for the first 100 s following the administration of rilmenidine was determined. Both concentration-dependence curves are depicted in Fig. 2B. The half-maximal effect of rilmenidine on $[Ca^{2+}]_i$ appeared to occur between concentrations of 0.01 and 0.1 m*M* when the response was measured as peak height above basal levels. A maximal effect was not observed when the response was measured as the average increase above basal level for the first 100 s.

Origin of the $[Ca^{2+}]_i$ increase evoked by rilmenidine and ATP

To determine if the increase in $[Ca^{2+}]_i$ evoked by rilmenidine was the result of influx, astrocytes were suspended in Ca^{2+}-free buffer supplemented with EGTA (1 m*M*) and exposed to rilmenidine. Under these conditions, the 0.1 and 10 m*M* rilmenidine responses were substantially reduced and the sustained elevation elicited by 10 m*M* rilmenidine was absent (Fig. 3A). The ATP response was also diminished in Ca^{2+}-free solution. Figure 3B demonstrates the peak heights of the increases in $[Ca^{2+}]_i$ induced by rilmenidine and ATP in Ca^{2+}-containing and Ca^{2+}-free solution. The unavailability of extracellular Ca^{2+} resulted in a peak height reduction of 80 and 67% for 0.1 and 10 m*M* rilmenidine, respectively. Similar reductions (91 and 72%) were seen when the peak heights during the initial 100 s were averaged for the 0.1 and 10 m*M* rilmenidine responses (data not shown).

The elevation of $[Ca^{2+}]_i$ induced by ATP was reduced by 77% when cells were suspended in Ca^{2+}-free buffer (Fig. 3B), suggesting that a large portion of the ATP response was due to Ca^{2+} influx and part of the response was due to release of Ca^{2+} from intracellular stores. This finding is consistent with that previously reported by Peuchen et al. (1996).

Ca^{2+} release from endoplasmic reticulum stores

Treatment of suspended astrocytes with the endoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin, evoked an immediate increase in $[Ca^{2+}]_i$ above basal level, followed by a smaller sustained elevation (Fig. 4A). Pretreating the cells with thapsigargin completely inhibited the Ca^{2+} response to rilmenidine (Fig. 4B). In contrast, ATP administered following thapsigargin consistently evoked a small increase in $[Ca^{2+}]_i$.

In Ca^{2+}-free medium, thapsigargin elicited a smaller response compared with that in Ca^{2+}-containing medium (Fig. 4C). The height of the initial peak of the thapsigargin response was reduced by 43% in Ca^{2+}-free medium, and the average elevation for the first

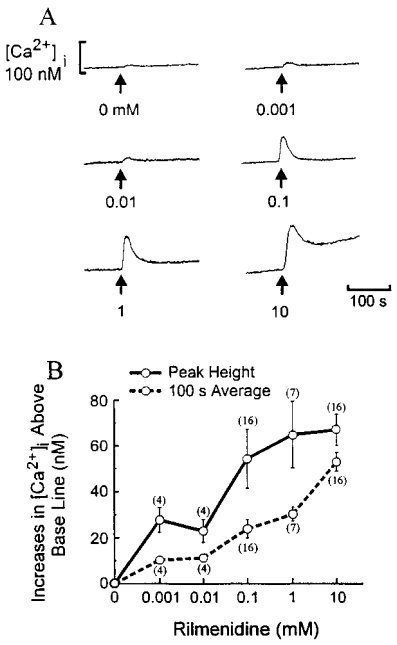

FIG. 2. A: Rilmenidine induces a concentration-dependent increase in $[Ca^{2+}]_i$ in suspended astrocytes. Arrows indicate addition of solvent (water, 0 m*M* rilmenidine) or rilmenidine (0.001–10 m*M*) into the cell suspension. **B:** Concentration–response curves for the increase in $[Ca^{2+}]_i$ induced by rilmenidine in suspended astrocytes. Solid line, peak height of response above basal level expressed in n*M*; broken line, average amplitude of response above basal level for the first 100 s expressed in n*M*. Data are means ± SEM, and the n values are stated in parentheses.

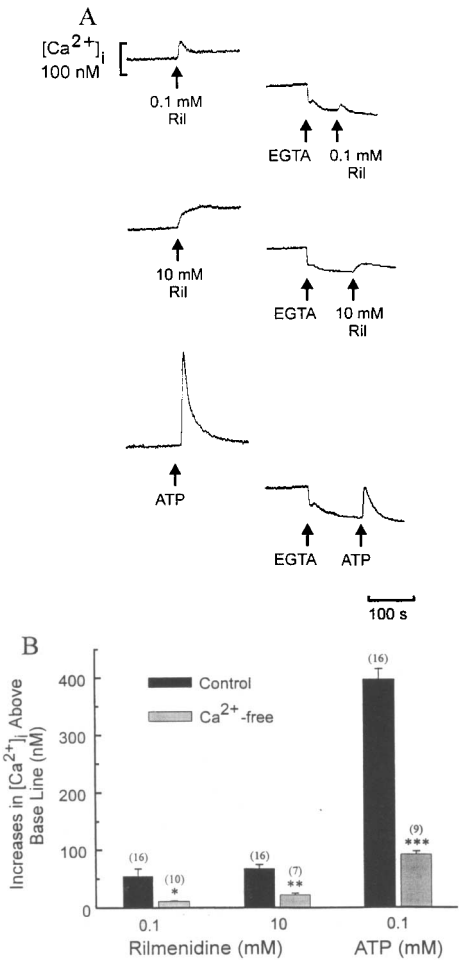

FIG. 3. A: Traces on the left are examples of elevations in $[Ca^{2+}]_i$ evoked by 0.1 and 10 m*M* rilmenidine (Ril) and by 0.1 m*M* ATP. Traces on the right are examples of increases in $[Ca^{2+}]_i$ evoked by the same agents when cells were suspended in Ca^{2+}-free buffer. The Ca^{2+} chelator EGTA (1 m*M*) was added where indicated. **B:** Peak height increases in $[Ca^{2+}]_i$ above basal levels induced by rilmenidine and ATP in Ca^{2+}-containing and Ca^{2+}-free buffer containing EGTA (1 m*M*). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$, compared with control response in Ca^{2+}-containing buffer. Data are means ± SEM, and sample size is shown in parentheses.

100 s was reduced by 41%. This suggests that there is some depletion of endoplasmic reticulum Ca^{2+} stores in Ca^{2+}-free medium. The sustained elevation in $[Ca^{2+}]_i$ induced by thapsigargin was substantially reduced (by 17–100%) in Ca^{2+}-free buffer (data not shown). This suggests that the sustained elevation in $[Ca^{2+}]_i$ following thapsigargin treatment is due to Ca^{2+} entry subsequent to release of Ca^{2+} from intracellular stores.

Pretreatment with an α_2-adrenergic receptor antagonist

The highly selective α_2-adrenergic antagonist rauwolscine was used to determine if the increase in $[Ca^{2+}]_i$ evoked by rilmenidine was mediated by α_2-adrenoceptors. Tanaka and Starke (1980) had shown previously that 0.01 m*M* rauwolscine maximally inhibits clonidine binding in rat cerebral cortex membranes. In our experiments, rauwolscine (0.01 m*M*) antagonized the increase in $[Ca^{2+}]_i$ elicited by the α_2-adrenergic agonist clonidine (Fig. 5A). However, rauwolscine had no effect on the responses elicited by either 0.1 or 10 m*M* rilmenidine. When mean peak increases above basal levels were examined, rauwolscine attenuated a large portion of the clonidine response, whereas it had no effect on the rilmenidine responses (Fig. 5B). When the mean increases in $[Ca^{2+}]_i$ for the initial 100 s were averaged, rauwolscine inhibited the response to clonidine by 75%, but had no effect on the rilmenidine response (data not shown).

$[Ca^{2+}]_i$ response to cirazoline

Cirazoline is an imidazoline receptor ligand (Angel et al., 1995). Cirazoline consistently evoked an increase in $[Ca^{2+}]_i$. The mean peak height of the cirazoline response above basal level was 134 ± 17 n*M* (n = 4), and the average height during the first 100 s was 108 ± 13 n*M* (n = 4). As cirazoline also has α_1-adrenergic actions (Van Meel et al., 1981; Ruffolo and Waddell, 1982), we examined whether the increase in

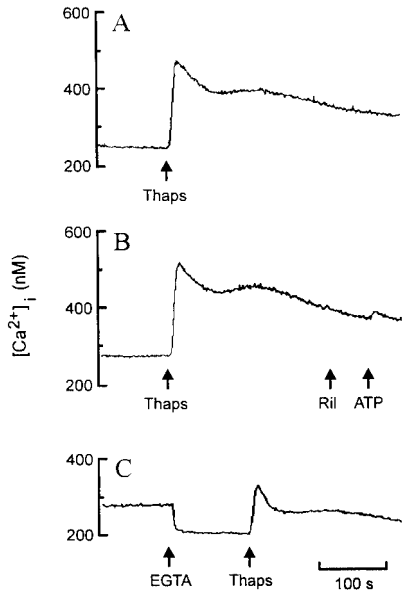

FIG. 4. A: Example of the increase in $[Ca^{2+}]_i$ in response to the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (Thaps; 20 μ*M*) when astrocytes were suspended in Na^+ buffer. **B:** Similar experiment as depicted in A, but including addition of rilmenidine (Ril; 10 m*M*) and ATP (0.1 m*M*) where indicated. **C:** An example of the elevation in $[Ca^{2+}]_i$ induced by thapsigargin (20 μ*M*) in Ca^{2+}-free Na^+ buffer containing EGTA (1 m*M*). Traces are respresentative of the complete loss of the rilmenidine response following thapsigargin treatment (n = 6), the reduction in the ATP response following thapsigargin treatment (n = 6), and the reduction in the peak increase and sustained plateau elicited by thapsigargin in Ca^{2+}-free Na^+ buffer (n = 4).

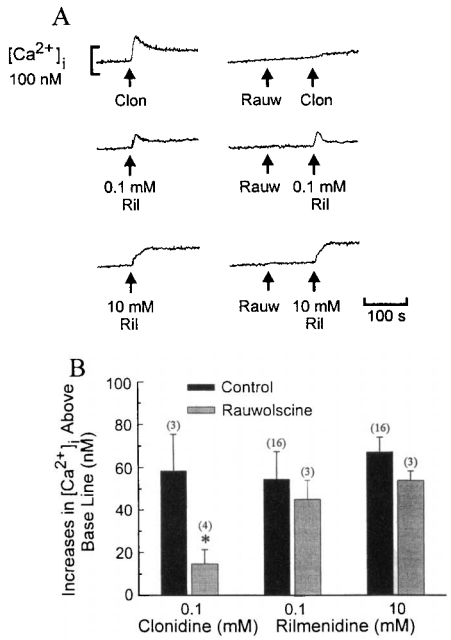

FIG. 5. A: Upper row: Examples of increases in $[Ca^{2+}]_i$ induced by clonidine (Clon; 0.1 m*M*) with and without prior administration of the α_2-adrenergic antagonist rauwolscine (Rauw; 0.01 m*M*) in astrocytes suspended in Na^+ buffer. **Middle row:** Sample traces of the increase in $[Ca^{2+}]_i$ in response to rilmenidine (Ril; 0.1 m*M*) with and without rauwolscine pretreatment. **Lower row:** Examples of the Ca^{2+} response to rilmenidine (10 m*M*) with and without rauwolscine pretreatment. **B:** Peak increases above basal levels in $[Ca^{2+}]_i$ induced by clonidine and rilmenidine in astrocytes in Na^+ buffer with and without rauwolscine (0.01 m*M*) pretreatment. $^*p < 0.05$, compared with control. Data are means ± SEM, and sample size is shown in parentheses.

$[Ca^{2+}]_i$ can be prevented by the α_1-adrenergic antagonist phentolamine. Phentolamine (0.1 m*M*) has been reported previously to inhibit cirazoline binding at α_1-adrenergic sites (Ruffolo and Waddell, 1982). Following phentolamine (0.1 m*M*) treatment, the $[Ca^{2+}]_i$ response to cirazoline was not significantly different. The peak height response was 184 ± 28 n*M* (n = 3), and the first 100 s response was 150 ± 35 n*M* (n = 3).

DISCUSSION

Rilmenidine causes a concentration-dependent elevation in astrocytic $[Ca^{2+}]_i$. An examination of the peak heights of these responses indicates that the maximum transient response occurs at ~0.1 m*M* rilmenidine. When Ca^{2+} was excluded from the extracellular medium, a small elevation in $[Ca^{2+}]_i$ was still observed, which indicates that rilmenidine evokes release of Ca^{2+} from intracellular stores. However, the magnitude of release from intracellular stores is likely underestimated, because suspending cells in Ca^{2+}-free buffer leads to depletion of intracellular Ca^{2+} stores. In this regard, the peak height of the thapsigargin response was markedly reduced in size when cells were suspended in Ca^{2+}-free buffer, suggesting that even brief exposure to Ca^{2+}-free buffer reduced endoplasmic reticulum Ca^{2+} stores. A similar finding by Murphy and Miller (1988) showed that the intracellular Ca^{2+} stores of hippocampal neurons become depleted in a time-dependent manner when extracellular Ca^{2+} concentration is reduced. To limit depletion of intracellular Ca^{2+} stores, our experiments were performed within minutes of suspending the astrocytes in Ca^{2+}-free buffer.

As the exclusion of extracellular Ca^{2+} leads to greatly reduced responses to rilmenidine, a portion of the increase in $[Ca^{2+}]_i$ evoked by rilmenidine appears to be the result of Ca^{2+} influx. This rilmenidine-induced Ca^{2+} influx may be due to the opening of plasma membrane Ca^{2+} channels activated by release of Ca^{2+} from intracellular stores [i.e., capacitative Ca^{2+} entry (Louzao et al., 1996)]. Capacitative Ca^{2+} entry has been characterized in mast cells (Hoth and Penner, 1992; Fasolato et al., 1993; Zweifach and Lewis, 1993). Furthermore, studies using endothelin (Marin et al., 1991), quisqualate (Glaum et al., 1990), and histamine (Fukui et al., 1991) support the existence of store-operated Ca^{2+} channels in astrocytes. It is possible that the Ca^{2+} influx induced by rilmenidine is mediated by capacitative Ca^{2+} entry. In many cell types, thapsigargin rapidly depletes endoplasmic reticulum Ca^{2+} stores, leading to the activation of capacitative Ca^{2+} entry (Takemura et al., 1989; Gouy et al., 1990; Mason et al., 1991). Following thapsigargin pretreatment, there was no response to rilmenidine. This is likely due to the following two reasons: first, rilmenidine did not induce release of Ca^{2+} from intracellular stores, because stores had been depleted; and second, rilmenidine did not induce Ca^{2+} influx, because capacitative Ca^{2+} entry was already maximally activated by thapsigargin.

Although previous studies have shown that increases in astrocytic $[Ca^{2+}]_i$ can be mediated via α-adrenergic receptors (Nilsson et al., 1991), it is likely that rilmenidine mediates its actions through a distinct receptor, likely the imidazoline receptor. This notion is based on the facts that the rilmenidine response was not attenuated by the α_2-adrenergic receptor antagonist rauwolscine and that another imidazoline receptor ligand, cirazoline, also causes elevation of $[Ca^{2+}]_i$ in astrocytes.

Clonidine induced an increase in astrocytic $[Ca^{2+}]_i$ that was likely mediated through α_2-adrenergic receptors, whereas the rilmenidine response was independent of α_2-adrenergic receptors. Although the clonidine response was similar in shape to the response to high concentrations of rilmenidine, the receptor through which the clonidine response was mediated appears to be distinct. α_2-Adrenoceptor activation was responsible for the majority of the increase in $[Ca^{2+}]_i$ induced by clonidine, as it was antagonized by rauwolscine. The portion of the clonidine response that could not be inhibited by rauwolscine may be the result of activation of another receptor type, perhaps imidazoline re-

ceptors. Rauwolscine did not antagonize the increase in $[Ca^{2+}]_i$ evoked by rilmenidine, indicating that rilmenidine activates a receptor distinct from α_2-adrenergic receptors.

Cirazoline, an imidazoline ligand and an α_1-adrenergic receptor agonist, caused an increase in astrocytic $[Ca^{2+}]_i$. This response to cirazoline could not be attenuated by pretreating the cells with phentolamine (an α_1-adrenergic receptor antagonist), suggesting that the action of cirazoline is independent of the α_1-adrenergic receptor. It is likely that cirazoline and rilmenidine, both imidazoline ligands, cause an increase in astrocytic $[Ca^{2+}]_i$ via a common receptor, possibly the imidazoline receptor.

The receptors mediating rilmenidine-induced Ca^{2+} mobilization are likely located on either the plasma membrane or endoplasmic reticulum. This does not correspond to the previous finding that astrocytic imidazoline receptors are localized exclusively on the outer mitochondrial membrane (Regunathan et al., 1993). The likelihood of another imidazoline receptor subtype (I_3) or a rilmenidine-binding site distinct from imidazoline and α_2-adrenergic receptors is supported by unidentified rilmenidine binding sites in the cerebral cortex (King et al., 1995). Other tissues expressing mitochondrial imidazoline receptors also express imidazoline receptors on the plasma membrane and on another unidentified cellular compartment (Lachaud-Pettiti et al., 1991; Tesson et al., 1991; Diamant et al., 1992).

The ability of rilmenidine to reduce the size of the infarction following an ischemic insult in the rat is dose-dependent (Maiese et al., 1992) and may correlate with rilmenidine's ability to stimulate astrocytic Ca^{2+} influx in a dose-dependent manner. Rilmenidine's neuroprotective effect may be related to its ability to stimulate astrocytic buffering of the neuronal environment from neurotoxic levels of Ca^{2+}. The concentrations of rilmenidine that induce increases in astrocytic $[Ca^{2+}]_i$ in this study are approximately the same as those of a therapeutic dose. For example, effective neuroprotection doses range from 0.5 to 2 mg/kg (Maiese et al., 1992), which correlates with blood concentrations of ~0.05–0.2 m*M*. These blood concentrations compare to rilmenidine concentrations of 0.01–0.1 m*M* required for the half-maximal increase in $[Ca^{2+}]_i$ in astrocytes.

ATP induces an increase in $[Ca^{2+}]_i$ within astrocytes via purinergic receptor stimulation (Nilsson et al., 1991; Peuchen et al., 1996). The rise in $[Ca^{2+}]_i$ results from both release of Ca^{2+} from the endoplasmic reticulum and Ca^{2+} influx (Peuchen et al., 1996). ATP was used in this study as a ligand known to increase astrocytic $[Ca^{2+}]_i$ as a comparison for the rilmenidine responses. ATP evoked increases in $[Ca^{2+}]_i$ in suspended astrocytes similar to those previously shown for single-cell measurements using plated astrocytes (Peuchen et al., 1996). Like the effect of rilmenidine, when astrocytes are suspended in Ca^{2+}-free medium, the response to ATP is reduced, suggesting that ATP induces release from Ca^{2+} stores and Ca^{2+} influx. However, unlike rilmenidine, ATP administered following thapsigargin pretreatment elicits a small increase in $[Ca^{2+}]_i$. This result suggests that ATP directly stimulates plasma membrane Ca^{2+} channels that are not the capacitative Ca^{2+} channels or that ATP induces the release of Ca^{2+} from stores other than endoplasmic reticulum.

Conclusion

Our study has clearly demonstrated that rilmenidine elevates $[Ca^{2+}]_i$ in primary cultures of rat cerebral astrocytes. The increase in $[Ca^{2+}]_i$ appears to be the result of release from endoplasmic reticulum stores and Ca^{2+} influx. This increase in $[Ca^{2+}]_i$ occurs by a mechanism independent of α_2-adrenoceptors. Astrocytes, and not neurons, within the cerebral cortex possess imidazoline receptors, and it has been suggested that these receptors are responsible for the neuroprotective effect of rilmenidine following an ischemic insult. Elevated levels of Ca^{2+} in nervous tissue following cerebral ischemia are detrimental to neurons (Siesjö and Bengtsson, 1989). The means of rilmenidine's neuroprotection following the setting of an ischemic insult may be related to its ability to induce astrocytic Ca^{2+} influx.

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