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Synergistic Effects in the α_1- and β_1-Adrenergic Regulations of Intracellular Calcium Levels in Striatal Astrocytes

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SUMMARY

1. Using indo-1 as a calcium fluorescent probe, we have observed the following in striatal astrocytes in primary culture.

2. The stimulation of α-adrenoceptors induces a rapid rise in cytosolic calcium resulting from an internal calcium mobilization followed by an external calcium influx (4-min duration).

3. The stimulation of β_1-adrenoceptors evokes only a slight internal calcium mobilization (90-sec duration).

4. The simultaneous stimulation of β_1- and α_1-adrenoceptors induces a more prolonged calcium influx (10 min). The latter phenomenon could explain the calcium-dependent synergistic effects of α_1 and β stimulation on cAMP production already described in the brain.

INTRODUCTION

Astrocytes from embryonic or newborn rats or mice in primary culture possess a broad array of receptors for neurotransmitters including β_1-, β_2-, and α_1-adrenergic receptors. β_1- and α_1-adrenoceptors have been investigated the most, their existence having been revealed by both autoradiographic (Burgess *et al.*, 1985; Lerea and McCarthy, 1989; McCarthy, 1983) and biochemical studies (Ebersolt *et al.*, 1981) and by measuring their positive coupling to adenylate cyclase (Ebersolt *et al.*, 1981) and phospholipase C, respectively (El Etr *et al.*,

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1989; Pearce *et al.*, 1985). The stimulation of α_1 receptors has been shown to depolarize astrocytes (Bowman *et al.*, 1987). In addition, numerous studies have demonstrated the role of β_1- and/or α_1-adrenoceptors in several effects of noradrenaline (NA), such as in phosphorylation of glial fibrillary acid protein (GFAP) (Pollenz and McCarthy, 1986) or vimentine (Browning and Sanders, 1981), glycogenolysis (Cummins *et al.*, 1983), release of taurine (Martin *et al.*, 1989; Shain *et al.*, 1989), and modifications of the astrocyte morphology (Narumi *et al.*, 1978).

Investigations performed on rat brain slices have indicated that the cAMP accumulation is increased synergistically when both β_1- and α_1-adrenoceptors are stimulated. The mechanism involved in this process has not been clearly established, but these synergistic effects require extracellular calcium (Schwabe and Daly, 1977). Recently, we have been able to observe these synergistic effects of the stimulation of β_1- and α_1-adrenoceptors on cAMP accumulation in striatal astrocytes from the mouse embryo in primary culture (Marin *et al.*, 1990). The present study has used primary cultures of striatal astrocytes from the mouse embryo, and the fluorescent calcium probe Indo-1 for single-cell microfluorimetry in order to determine whether the stimulation of β_1- and α_1-adrenoceptors are both associated with modifications in cytosolic calcium levels and whether the synergistic effects of these responses can be demonstrated. Moreover, data obtained in peripheral tissues have suggested that both classes of adrenoceptors are able to mediate an increase in cytosolic calcium (Han *et al.*, 1987, Birnbaumer *et al.*, 1989). There is also some evidence for changes in the cytosolic concentration of free ionized calcium in astrocytes under the stimulation of α_1-adrenergic receptors (Enkvist *et al.*, 1989).

MATERIALS AND METHODS

Glial Primary Cultures. Striatal astrocytes from 16-day-old Swiss mouse embryos were dissociated mechanically in serum-free medium. Cells (10^6 per 100-mm dish) were plated on glass slides previously coated with poly-L-ornithine (1.5 μg/ml, Sigma) and placed in the culture dishes. The culture medium was composed of Dulbecco's modified essential medium (DMEM) and F-12 nutrient (GIBCO, Europe) supplemented with glucose (33 m*M*), glutamine (2 m*M*), $NaHCO_3$ (3 m*M*), Hepes (5 m*M*), and 10% NU-Serum (Collaborative Research). Cells were cultured at 37°C for 18 to 20 days in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed on day 12; cytosine arabinoside (1 μ*M* Sigma) was added in order to avoid the formation of cell multilayers. In these conditions, more than 95% of cells were stained by the indirect immunofluorescence technique using a rabbit antibody against glial fibrillary acid protein (GFAP) (Dakopatts, Denmark). The cultures were free of microglial cells, since no staining was observed using monoclonal anti-mouse macrophage antibody (anti-MAC 1) (Serotec, France).

Optical Device for Indo-1. Cytosolic Ca^{2+} measurement was carried out in dual-emission microfluorimetry with the probe Indo-1. Cells loaded with the

fluorescent probe were excitated through a 40 × oil-immersion fluorine objective using a 75-W xenon light, neutrally attenuated to avoid bleaching and filtered at 360 nm with a 10-nm-wide interferential filter. Excitation and emission lights were separated by a 380-nm dichroic long-pass filter. Emitted spectra was then divided in two halves by a 455-nm dichroic long-pass filter. Two discriminant bands were selected by interferential filters at 400–410 and 470–490 nm from the two halves of the Indo-1 emission spectra, photon signals being amplified and estimated by photometers. The fluorescence ratio F_{405}/F_{480}, which is independent of the probe concentration, was directly calculated from both simultaneous signals.

All optical and photometers were obtained from Nikon–France, Nikon Europe b.v., and Nikon Instruments Japan. The fluorescent probe was purchased from Molecular Probe Inc.

Biological Parameters and Calibration. Under the conditions used, the spontaneous fluorescence of astrocytes was negligible. According to the equation described by Grynkiewicz *et al.* (1985), cytosolic Ca^{2+} concentrations were calculated from the fluorescence ratio R measured at 400–410 and 470–490 nm, respectively, and a 250 nM Kd of Indo-1 for ionized calcium: $[Ca^{2+}] = K_d \times (F_{480_f}/F_{480_b}) \times (R\text{-}R_{\min})/R_{\max}\text{-}R)$, in which F_{480_f} is the fluorescence of free Indo-1 and F_{480_b} the fluorescence of Indo-1 bound to Ca^{2+}; R is the ratio between the fluorescence measured at 405 nm and that at 480 nm; and R minimum ($R_{\min}$) and R maximum ($R_{\max}$) were determined in the presence of ionomycine (10 μM) and either EGTA (2 mM) or $CaCl_2$ (2 mM), respectively.

Loading, Perfusion, and Pharmacological Superfusion. Astrocytes in a confluent monolayer on the glass slide were loaded during 60 min with 12 μM of the cell permeant Indo-1 acetoxymethylester (Indo-1/AM) in "perfusion saline Hepes buffer." After loading, the glass slide was disposed in a thermostable perfusion chamber. The cells were exposed to various substances (at indicated concentrations) using a multichannel cell superfusion device allowing the superfusion medium to be changed in less than 1 sec. Pharmacological substances to be tested were dissolved in the perfusion saline Hepes buffer containing Hepes (20 mM), glucose (5.5 mM), NaCl (145 mM), KCl (5.5 mM), $MgCl_2$ (0.9 mM), and $CaCl_2$ (1.2 mM). In external calcium-free experiments, calcium was omitted and EGTA (5 mM) added.

In order to determine optimal concentrations of the pharmacological substances tested, dose–response curves were made in preliminary experiments. For each compound, the minimal concentration allowing the maximal effect was adopted.

Statistics. To test for statistical significance, means were compared according to the Fischer–Student test modified for samples less than 30, with calculation of a common variance, as described by Schwartz (1986).

RESULTS

Effects of the Stimulation of α_1-Adrenergic Receptors on the Cytosolic Concentration of Calcium in Striatal Astrocytes. In the presence of extracellular calcium, in all the cells tested, ($n = 20$), methoxamine (25 μM), a selective

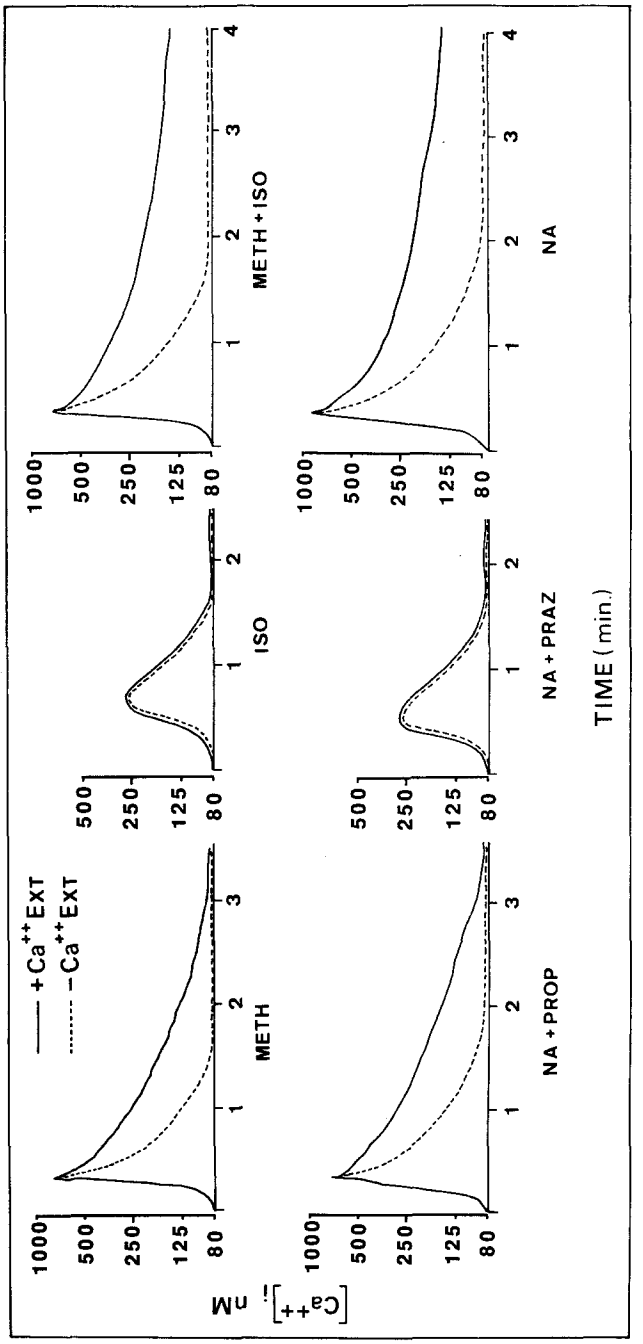

Fig. 1. Regulation by α_1 and β_1-adrenoceptors of the cytosolic Ca^{2+} concentration. Dependency on the presence of external calcium. Cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ (n*M*) variations were measured in the absence (----) or presence (——) of extracellular calcium, 1.2 m*M* (Ca^{2+} Ext), in response to methoxamine, 25 μM (METH), isoproterenol, 10 μM (ISO), and noradrenaline, 25 μM (NA), alone or in the presence of prazosin, 100 n*M* (PRAZ), or propranolol, 100 n*M* (PROP). The data shown were obtained on single cells and represent typical observations. For each treatment, similar experiments repeated on five other cells provided similar results.

agonist of α_1-adrenergic receptors, induced a reproducible increase in the cytosolic Ca^{2+} concentration (Fig. 1). This response was composed of a brief increase occurring in about 10 sec, reaching a maximal value of 730 n*M*, followed by a progressive decline, with the basal level being recovered in 3 to 5 min (see also Fig. 4). The methoxamine (100 μM)-evoked response was completely abolished by the combined addition of prazosin (100 n*M*) while it remained unaffected in the presence of propranolol (100 n*M*) (data not shown).

The response evoked by methoxamine was modified markedly when extracellular calcium was removed and EGTA (5 m*M*) added into the perfusion medium. The initial rising phase was similar to that observed in the presence of extracellular calcium, but the change in the concentration of cytosolic Ca^{2+} was of a much shorter duration since recovery to basal level occurred in about 90 sec (Fig. 1).

Results obtained in the presence or absence of extracellular calcium suggest that methoxamine acts first by mobilizing calcium ions from their intracellular stores (S1 response) and then by stimulating the influx of external calcium (S2 response). The role of external calcium was further demonstrated by removing external calcium about 2 min after the initial peak of the methoxamine-evoked response. This accelerated dramatically the decline of cytosolic Ca^{2+} (Fig. 2). In

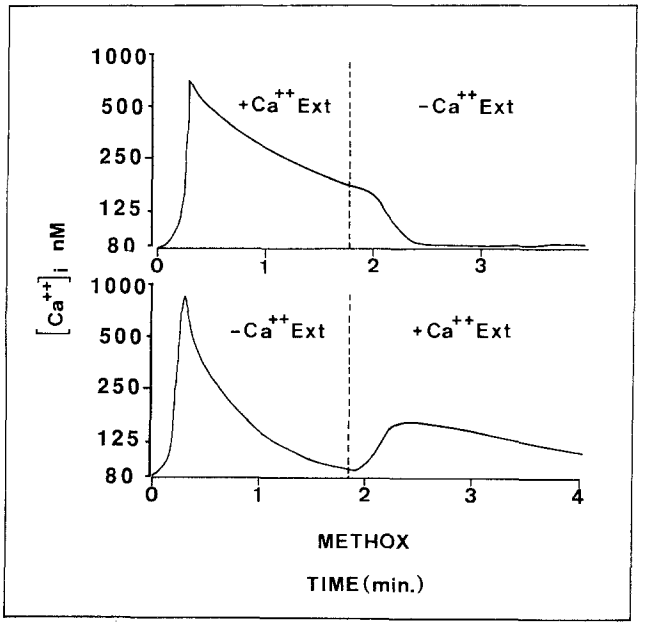

Fig. 2. Effects of a sequential withdrawal or addition of external calcium on the cytosolic Ca^{2+} concentration during stimulation of α_1-adrenoceptors by methoxamine. Dashed lines indicated the time of withdrawal (upper part) or addition (lower part) of external calcium (1.2 m*M*). Methoxamine was applied at a concentration of 25 μM. Typical results obtained on single cells are represented. Similar observations were made in five other experiments.

addition, when striatal astrocytes were exposed to methoxamine first in the absence and then in the presence of external calcium, added at the end of the S1 response, a second cytosolic Ca^{2+} increase was observed.

As shown in Fig. 3, the methoxamine-evoked response could be repeated in both the presence and the absence of external calcium (Fig. 3). However, more than 15 repetitions were obtained in the presence of external calcium, but no more than 5 when calcium was removed. In addition, the amplitude of the initial peak decreased progressively following each successive application of the α_1-agonist made at 3-min intervals, this phenomenon being more pronounced in the absence than in the presence of extracellular calcium (Fig. 3).

Since preliminary experiments indicated that the NA-evoked response was not affected by the α_2-antagonist yohimbine (10 μM), α_1-adrenoceptors were also selectively stimulated by exposing striatal astrocytes to a simultaneous application of NA (25 μM) and propranolol (10 μM). Under these conditions, changes in cytosolic Ca^{2+} levels observed in the presence or absence of external calcium were identical to those induced by methoxamine alone (Fig. 1).

Effects of the Stimulation of β-Adrenergic Receptors on the Cytosolic Concentration of Calcium in Striatal Astrocytes. Surprisingly, isoproterenol (10 μM), a nonselective agonist of β_1- and β_2-receptors modified the levels of cytosolic Ca^{2+} in striatal astrocytes. However, the reproducibility and the pattern of the response differed from those observed with methoxamine. Only half of the

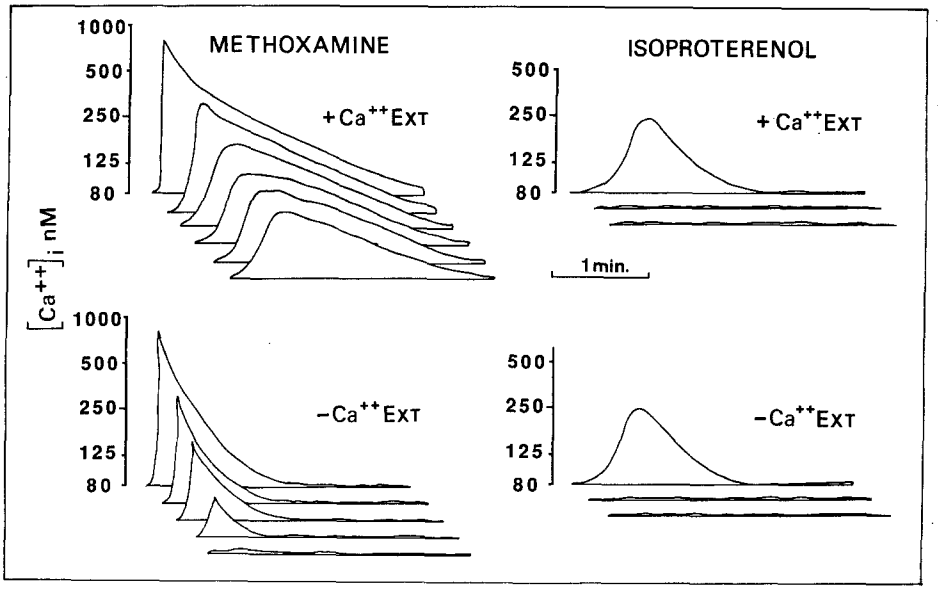

Fig. 3. Effects of repeated stimulations of α_1- or β_1-adrenoceptors on cytosolic Ca^{2+} concentrations during stimulation of α_1-adrenoceptor by methoxamine. The application of methoxamine 25, μM, or isoproterenol 10 μM, was, repetitively, made on the same cell during 4 min, each application being separated by 3-min intervals. Experiments were carried out in the absence or presence of external calcium 1.2 mM (Ca^{2+} Ext). Five other independent experiments provided similar results.

cells tested ($n = 30$) responded to isoproterenol. The effect was of a shorter duration (about 90 sec) and characterized by a smooth peak occurring after 20 sec and reaching a maximal average value of 260 nM (Figs. 1 and 4). Propranolol (10 μM) antagonized completely the isoproterenol-evoked response (data not shown). In contrast to that found with methoxamine, the effect of isoproterenol was not affected by removal of external calcium (Fig. 1). Therefore, only a mobilization of Ca^{2+} from intracellular stores seem to occur under stimulation of β-adrenoceptors.

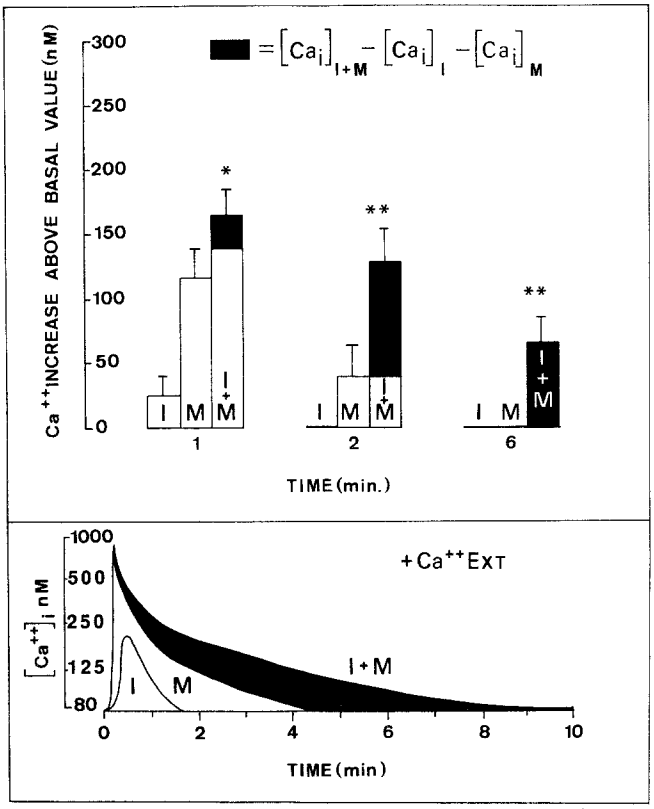

Fig. 4. Time course of the synergistic effects of the coactivation of α_1- and β_1-adrenoceptors on striatal astrocytes. In the upper part of the figure, the increases above basal level in the cytosolic concentrations of Ca^{2+} were indicated on the ordinate at different times (1, 2, and 6 min) after the addition of methoxamine, 25 μM (M), isoproterenol, 10 μM (I), or both (I + M) in the presence of external calcium (1.2 mM). Values are the mean ± SE of a single determination obtained on 20 different cells. (*) $P < 0.05$; (**) $P < 0.01$. Significantly different from the sum of values obtained in the presence of each agonist alone, isoproterenol or methoxamine. In the lower part of the figure, complete and typical kinetic variations in cytosolic Ca^{2+} concentrations observed in the presence of isoproterenol, 10 μM, or methoxamine, 25 μM, or both are illustrated, data being obtained on single cells.

β_1- but not β_2-adrenergic receptors are involved in the isoproterenol-induced modifications of cytosolic Ca^{2+}. Hence, salbutamol (10 m*M*), a selective β_2-agonist, was without effect, although at the concentration used, it stimulates the activity of adenylate cyclase on membranes from striatal astrocytes (unpublished observations).

β_1-adrenoceptors were also stimulated selectively by NA (25 μM) applied in the presence of prazosin (100 n*M*). The response was closely similar to that of isoproterenol, in either the presence or the absence of extracellular calcium (Fig. 1). The response evoked either by isoproterenol alone (Fig. 3) or by the combined application of NA and prazosin could not be repeated.

Effects of the Simultaneous Activation of α_1- and β_1-Adrenergic Receptors. In the absence of external calcium, the combined application of methoxamine (25 μM) and isoproterenol (10 μM) triggered a response closely similar to that found with methoxamine alone: a rapid rise in cytosolic free calcium concentration of short duration.

In contrast, in the presence of external calcium, the coactivation of β_1- and α_1-adrenergic receptors induced a response which was much more pronounced than those observed with methoxamine or isoproterenol alone. These synergistic effects, seen in all cells tested ($n = 20$), were prominent as soon as 1 min after the onset of the combined application of the α_1- and β_1-agonists (Figs. 1 and 4). The cytosolic concentration of free calcium was indeed significantly higher at 1, 2, and 6 min compared to the summation of the responses obtained separately with α_1- and β_1-agonists (Fig. 4).

Additional evidence for the synergistic effects of isoproterenol and methoxamine was obtained in complementary experiments in which each agonist was applied for a long period and the other at the end of the response triggered by the first one. As illustrated in Fig. 5, in the presence of the other agonist, the methoxamine- or isoproterenol-induced elevations of cytosolic Ca^{2+} were of much longer duration and amplitude than the response obtained with each agonist alone. Moreover, isoproterenol potentiated the methoxamine-evoked response even in astrocytes that did not respond to isoproterenol alone (Fig. 5).

Experiments performed with NA (25 μM) confirmed both that the combined stimulation of α_1- and β_1-adrenoceptors triggers sustained modifications in cytosolic free calcium levels and that this phenomenon occurs only in the presence of extracellular calcium (Fig. 1). Both removal of external calcium and addition of prazosin 2 min after the onset of the NA-evoked response were associated with a rapid decline in cytosolic free Ca^{2+} levels. As expected, for a synergistic action, the blockade of either the α_1- or the β_1-adrenergic receptors by prazosin or propranolol, respectively, resulted in a shortening of the NA-evoked response. The addition of dihydropyridines (nitrendipine or nifedipine, 10 μM) during the S2 phase did not affect the pattern of the NA-evoked response (Fig. 6). Similar results were obtained when dihydropyridines were added from the onset of the NA stimulation (data not shown). This suggests that classical voltage-dependent calcium channels are not involved in the NA-evoked influx of calcium.

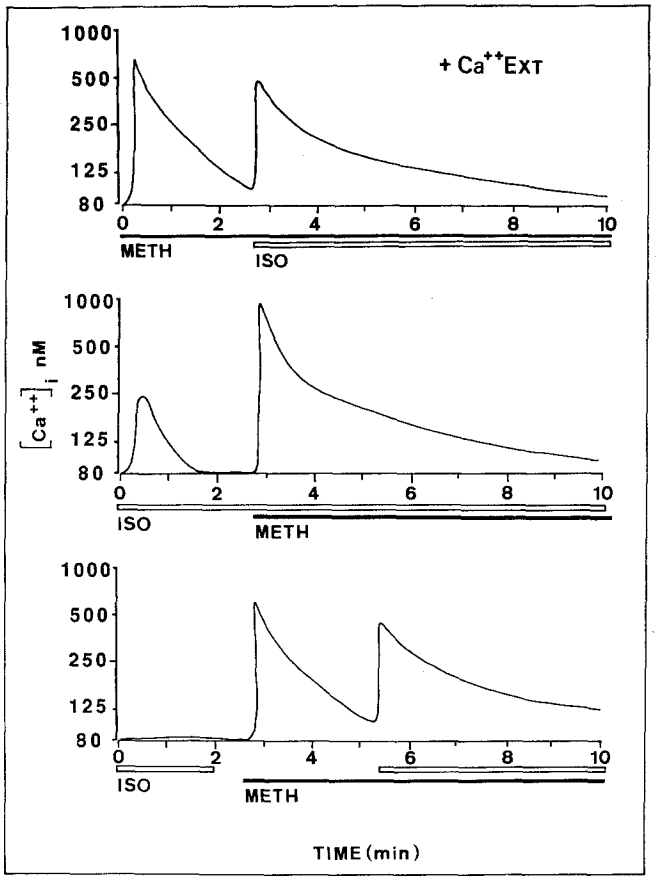

Fig. 5. Effects of sequential additions of α_1- and β_1-agononists on cytosolic Ca^{2+} concentrations in striatal astrocytes. Methoxamine, 25 μM (METH), and/or isoproterenol, 10 μM (ISO), were added according to the sequence indicated in the figure. As illustrated in the lower part of the figure, the synergistic effects of methoxamine and isoproterenol were observed in cells which did not respond to an initial application of isoproterenol alone. Data illustrated represent typical findings obtained in single cells and repeated at least on five other cells with qualitatively similar results.

DISCUSSION

Using the Indo-1 method, a highly sensitive procedure to detect changes in intracellular free calcium levels, we have shown that the stimulation of either α_1- or β_1-adrenergic receptors increases the cytosolic Ca^{2+} in primary cultures of striatal astrocytes from embryonic mice. However, different mechanisms are involved in the α_1- and β_1-adrenergic-mediated responses. Furthermore, we have demonstrated that coactivation of β_1-adrenoceptors prolonged the response triggered by the stimulation of α_1-receptors and that these synergistic effects require the presence of extracellular calcium.

The stimulation of α_1-adrenoceptors by either the selective α_1-agonist

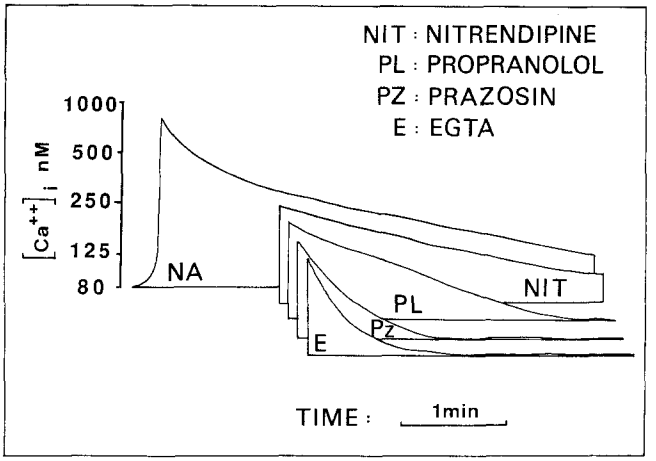

Fig. 6. Pharmacological characteristic concentrations of the calcium influx induced by noradrenaline in striatal astrocytes. Nitrendipine, 10 μM (NIT), propranolol, 10 μM (PL), prazosin, 100 nM (PZ), or EGTA, 5 mM (E), were added 1.5 min after the addition of noradrenaline, 25 μM (NA). The data shown correspond to typical variations in cytosolic concentrations of Ca^{2+} obtained in single cells and repeated five times at least with qualitatively similar results.

methoxamine or the combined application of NA and the β_1-antagonist propranolol rapidly enhanced cytosolic Ca^{2+} levels by two mechanisms: (1) an initial response linked to a mobilization of calcium from intracellular stores (S1 response) and (2) an influx of external calcium responsible for the distal phase of the response (S2 response). Since it is well established that the stimulation of α_1-receptors activates phospholipase C in astrocytes, the mobilization of intracellular calcium can be attributed to the effect of inositol trisphosphate (IP_3) formed on calcium stores (Berridge and Irvine, 1989). Studies made at the periphery have indicated that distinct α_1-receptors are responsible for the influx of external calcium through their action on voltage-operated calcium channels (VOCCs) (Han *et al.*, 1978). Presently, we cannot conclude whether or not distinct subtypes of α_1-receptors are involved in the effects on striatal astrocytes. Indeed, L-type VOCCs do not seem to contribute to the α_1-receptor-mediated influx of external calcium in striatal astrocytes since neither nitrendipine nor nifedipine, two dihydropyridines known to block these channels (McCleskey *et al.*, 1987), modified the pattern of the methoxamine-evoked response. This agrees with recent reports which have indicated that under control conditions, L-type VOCCs cannot be detected on astrocytes in primary cultures (Barres *et al.*, 1989). Indeed, the induction of these channels requires a 15- to 20-min exposure of the cells to a permeant cAMP analogue or to agents increasing intracellular levels of cAMP. More critically, culture of the cells for 48 hr with a permissive serum distinct from the nonpermissive serum used in our study is necessary; T-type VOCCs present on astrocytes (McCleskey *et al.*, 1987), which can be activated under stimulation, could be involved in the influx of external calcium. Indeed, astrocytes are

depolarized under stimulation of α_1-receptors, and this results mainly from the blockade of K^+ channels linked to the activation of protein kinase C (Enkvist *et al.*, 1989). Whatever the mechanism involved, our results agree with those of others who used the $^{45}Ca^{2+}$ or the Fura-2 methods in order to determine the role of α_1-receptors in the control of cytosolic free calcium levels in primary cultures of cortical astrocytes from the newborn rat (Enkvist *et al.*, 1989). However, in these studies, phenylephrine was used as a selective α_1-agonist; this can be challenged since phenylephrine enhanced cAMP levels in astrocytes and this effect is partly blocked by the β-antagonist propranolol (Marin *et al.*, 1990).

In heart, in addition to their coupling to adenylate cyclase, β-adrenergic receptors are linked to calcium channels through a G_S protein (Brown and Birnbaumer, 1988, Birnbaumer *et al.*, 1989). To our knowledge, we show here for the first time that the stimulation of β_1-receptors induced either by isoproterenol or by the combined application of NA and prazosin transiently increases the cytosolic Ca^{2+} level in astrocytes. β_1-adrenergic receptors are involved since salbutamol, a β_2-selective agonist, was without effect. However, the mechanism responsible for this effect in the striatal astrocytes seems to differ from that described in heart since the β_1-mediated response was independent of external calcium. Studies made in aortic smooth muscle cells have suggested that intracellular calcium channels located on the reticulum and linked to IP3 receptors could also be kept open after phosphroylation through protein kinase A (Tawada *et al.*, 1987). Such a cAMP-dependent process triggered by the activation of β_1-adrenoceptor could also be involved in striatal astrocytes. Nevertheless, coaddition of a cAMP permeant analogue, 8 bromo-cAMP (up to 100 μM), with methoxamine did not reproduce the synergistic effect of isoproterenol (Marin *et al.*, 1990). The pattern of mobilization of calcium from intracellular stores linked to the stimulation of β_1-receptors differed from that observed under stimulation of α_1-adrenoceptors. This suggests the contribution of different pools of calcium. Indeed, in the absence of external calcium, the isoproterenol-evoked response was of a lower amplitude (250 nM) and shorter duration (90 sec) than that triggered by methoxamine. In addition, the isoproterenol- evoked response could not be repeated when successive 3-min applications of the β_1-agonist were made at 3- to 15-min intervals. This is in contrast to that observed with methoxamine even in the absence of extracellular calcium. It could be argued that β_1-receptors are desensitized more rapidly than α_1-adrenoceptors. Alternatively, it could be proposed that the intracellular pool of calcium having been depleted during the first stimulation, a second one may remain ineffective even in the absence of a receptor desensitization process.

The most striking phenomenon of our experiments is the synergistic effects of α_1- and β_1-agonists on cytosolic free calcium levels. Indeed, in the presence of isoproterenol, the influx of calcium induced by methoxamine was not only increased but also prolonged. The synergistic effects of the stimulation of α_1- and β_1-adrenoceptors on cytosolic Ca^{2+} were reproduced by NA. The critical role of external calcium in this process was demonstrated in several ways. The synergistic effects of α_1- and β_1-agonists did not occur in the absence of extracellular calcium and the prolonged response seen with NA alone was interrupted immediately

after removal of external calcium. Propranolol and prazosin accelerated the decline of cytosolic Ca^{2+} to basal levels when added 2 min after the onset of NA application. This further demonstrated the contribution of both α_1- and β_1-adrenergic receptors in the secondary long-term phase of the NA response. Similarly to what had been noted with methoxamine alone, L-type VOCCs seem not to be involved in the synergistic effects of α_1- and β_1-mediated responses since the dihydropyridines, added at any time during the stimulation, did not modify the pattern of the NA-evoked response. Moreover, the depolarization of astrocytes by 50 m*M* KCl scarcely induced an increase in cytosolic calcium (data not shown). Most of the cells which responded to α_1-agonists were insenstive to 50 m*M* KCl application, indicating that the α_1-evoked response did not involve voltage-sensitive calcium channels.

Precise information on the molecular events intervening in the synergistic effects observed cannot yet be provided. However, it should be noted that (1) the synergistic effects of isoproterenol and methoxamine on cytosolic Ca^{2+} levels were still observed when methoxamine was applied at the end of the isoproterenol-evoked response, although the effect of isoproterenol alone cannot be repeated even in the presence of extracellular calcium; (2) the synergy was seen even in cells in which isoproterenol alone did not affect intracellular stores of Ca^{2+} (half of the cells tested); and (3) finally, the isoproterenol-evoked response was considerably amplified in the presence of methoxamine when the β_1-agonist was applied at the end of the response evoked by the α_1-agonist. These results indicate that pronounced and rapid desensitization of both α_1- and β_1-adrenoceptors does not occur under our conditions or suggest that such desensitization may even be prevented under their coactivation.

The prolonged influx of calcium linked to the coactivation of α_1- and β_1-adrenoceptors could easily explain the synergistic effects of α_1- and β_1-agonists on cAMP production observed in brain slices of adult animals (Schlutz and Daly, 1973) or in astrocytes from the mouse embryo (Marin *et al.*, 1990). The latter phenomenon is indeed dependent on the influx of external calcium (Schwabe and Daly, 1977). It has been proposed that prostaglandins are involved in the synergism in the production of cAMP seen in brain slices (Partington *et al.*, 1980). Prostaglandins synthesis could be linked to the prolonged influx of calcium due to the coactivation of α_1- and β_1-adrenoceptors.

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