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Interaction of Subtype-Selective Antagonists with α_1-Adrenergic Receptor-Mediated Second Messenger Responses in Rat Brain

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SUMMARY

The selective antagonists (+)-niguldipine and 5-methylurapidil (5-MU) were used to more clearly identify the α_1-adrenergic receptor subtypes involved in second messenger responses in slice and culture preparations of rat brain. The α_1-adrenergic receptor activating [^{3}H]inositol phosphate (InsP) formation in neocortical and hippocampal slices appeared to have mixed characteristics. Although the low potency of (+)-niguldipine indicated involvement of the α_{1B} subtype, 5-MU had an α_{1A}-like potency at this subtype. (+)-Niguldipine did not inhibit the α_1 receptor-mediated potentiation of the cAMP response to either isoproterenol or adenosine in cortical slices, even at high concentrations. 5-MU inhibited both cAMP responses, although this inhibition appeared noncompetitive. Thus, these receptors are clearly different from those mediating InsP formation. In primary glial cultures, (+)-niguldipine also had a low potency in blocking norepinephrine-stimulated [^{3}H]InsP formation, consistent with involvement of the α_{1B} subtype. However, both 5-MU and WB 4101 had high potencies in blocking this response, suggesting involvement of the α_{1A} subtype. Inactivation of the α_{1B} subtype by pretreatment of cultures with chloroethylclonidine did not increase the potencies of any of these antagonists. The inhibition by 5-MU and WB 4101 was competitive in both control and chloroethylclonidine-pretreated cultures, whereas the inhibition by (+)-niguldipine was primarily noncompetitive. The use of these more selective antagonists shows that the current α_{1A}/α_{1B} subclassification scheme is inadequate to identify the receptors mediating these responses. None of the responses were blocked by (+)-niguldipine with the high potency expected at the α_{1A} subtype, although all InsP responses were blocked by 5-MU with a relatively high (α_{1A}-like) potency. In addition, very low affinity and noncompetitive effects of (+)-niguldipine were observed. These data raise the possibility of additional subtypes of α_1-adrenergic receptors or as yet unidentified functional interactions between known subtypes.

Two pharmacologically distinct subtypes of α_1-adrenergic receptors can be distinguished with competitive antagonists and alkylating agents. The α_{1A} subtype was defined by its high affinity for WB 4101 and phentolamine (1, 2) and its insensitivity to alkylation by CEC (3, 4), whereas the α_{1B} subtype is potently inactivated by CEC and has a lower affinity for WB 4101 (1–4). These receptor subtypes have different tissue distributions and have been suggested to be linked to signaling mechanisms with differential involvement of extracellular Ca^{2+} (2–7).

Recently, additional antagonists that discriminate between α_1-adrenergic receptor subtypes have been reported. Both 5-MU (8, 9), a serotonin (5-hydroxytryptamine$_{1A}$) receptor antagonist, and (+)-niguldipine (10, 11), a dihydropyridine calcium channel antagonist, have been reported to be highly selective for the α_{1A} subtype. These drugs have been reported to have a substantially greater selectivity than WB 4101 and phentolamine.

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It has recently been reported that (+)-niguldipine shows a remarkable selectivity between the α_1-adrenergic receptors linked to two different biochemical responses in slices of rat brain. (+)-Niguldipine blocks the increase in InsPs caused by α_1-adrenergic receptor activation with a relatively low affinity, indicative of the α_{1B} subtype (12). However, it is almost completely ineffective in blocking the potentiation of cAMP accumulation caused by α_1-adrenergic receptor activation in the presence of isoproterenol (12). This raises the possibility of additional α_1-adrenergic receptor subtypes in rat brain, with differential affinities for (+)-niguldipine.

In this manuscript, we report the interaction of (+)-niguldipine and other subtype-selective antagonists with InsP and cAMP responses to α_1-adrenergic receptor activation, in slices and cultures from rat brain. We hoped to determine whether the use of these more selective drugs could clarify which responses were mediated by the pharmacologically defined α_{1A} and α_{1B} subtypes.

Experimental Procedures

Materials. Timed pregnant or 150–250-g male Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). The following chemicals

ABBREVIATIONS: CEC, chloroethylclonidine; KRB, Krebs' Ringer bicarbonate buffer; InsP, inositol phosphate; PBZ, phenoxybenzamine; BIM, alkylating pindolol; NE, norepinephrine; 5-MU, 5-methylurapidil; DMEM, Dulbecco's modified Eagle's medium; CBSd, calf bovine serum defined.

were used: DMEM and calf bovine serum (Hyclone Laboratories, Logan, UT); twice crystallized trypsin (Worthington Biochemicals, Freehold, NJ); CEC, WB 4101, and 5-MU (Research Biochemicals Inc., Natick, MA); (+)-niguldipine (Byk Gulden, Konstanz, FRG); prazosin (Pfizer, Groton, CT); phentolamine mesylate (CIBA-Geigy, Summit, NJ); poly-L-lysine, amphotericin B, streptomycin, penicillin G, deoxyribonuclease I, yohimbine HCl, isoproterenol bitartrate, adenosine, and nifedipine (Sigma Chemical Co., St. Louis, MO); PBZ HCl (Smith Kline & French, King of Prussia, PA); BIM (Dr. J. Pitha, National Institute of Aging); [^{3}H]inositol (10–20 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO); and [2,8-^{3}H]adenine (10–25 Ci/mmol; DuPont NEN, Boston, MA).

Slice preparation. Rats were killed by decapitation and the brains were removed. Neocortex or hippocampus was dissected and cross-chopped into 350 × 350 μm trapezoids on a McIlwain tissue chopper. Slices were dispersed in KRB (in mM: NaCl, 120; KCl, 5.5; $CaCl_2$, 2.5; $NaHCO_3$, 20; glucose, 11; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $CaNa_2$ EDTA, 0.029) previously equilibrated with 95% O_2/5% CO_2 at 37°. The KRB was decanted and the slices were resuspended in KRB and incubated for 15 min at 37° under O_2/CO_2.

cAMP accumulation in slices. The [^{3}H]adenine prelabeling technique of Shimizu *et al.* (13) was used to monitor cAMP accumulation in slices, as previously described (14). Briefly, slices were decanted and incubated in KRB containing [^{3}H]adenine (1 μCi/ml) and 3 μM unlabeled adenine, for 40 min at 37° under O_2/CO_2. The slices were then collected on a nylon mesh and washed with 500 ml of cold KRB. In experiments where alkylating agents were used, slices were resuspended in 50 ml of KRB and incubated with or without the indicated concentrations of alkylating agents for 10 min. Slices were again collected on nylon mesh and washed with another 500 ml of cold KRB. The slices were resuspended in KRB, and 75 μl of gravity-packed slices were added to the reaction tubes containing KRB and appropriate drugs, in a final volume of 1 ml. Tubes were incubated for 15 min at 37° under O_2/CO_2, and the reaction was stopped with 0.1 ml of 77% trichloroacetic acid. Samples were homogenized, carrier was added, incorporations were calculated, and [^{3}H]cAMP was isolated by sequential Dowex and alumina chromatography, as described (14).

[^{3}H]InsP accumulation in slices. [^{3}H]InsP accumulation was measured in cortical slices as previously described (15). Briefly, 75 μl of gravity-packed slices were incubated for 2 hr in the presence of [^{3}H] inositol, with or without 100 μM NE, in KRB containing 10 mM LiCl. Reactions were stopped by addition of chloroform/methanol, and [^{3}H] InsPs were isolated by anion exchange chromatography.

Cell cultures. Primary glial cultures were prepared from 1-day-old rat brains by the method of Raizada (16), as described by Wilson *et al.* (17). Brains were removed and placed in an isotonic salt solution containing 0.25 μg/liter amphotericin B, 100 μg/liter streptomycin, and 100 units/liter penicillin, pH 7.2. Pia mater and blood vessels were removed, and the brains were chopped into approximately 2-mm chunks. Minced tissue from 7–14 brains was suspended in 25 ml of 0.25% trypsin (w/v) in an isotonic salt solution and placed in a 37° shaking water bath for 6 min. Deoxyribonuclease I (160 μg) was then added to the cell suspension, which then continued shaking another 6 min. Dissociated cells were collected in 10 ml of DMEM containing 10% CBSd. Undissociated tissue was triturated several times, and all cells were washed with 40 ml of DMEM/CBSd and centrifuged for 10 min at 1000 × *g*. Recoveries were normally 40–50 × 10^6 cells/brain. Cells were then resuspended in DMEM/CBSd, and 4 ml (2 × 106 cells/ml) were plated in Falcon tissue culture dishes (60 mm) precoated with poly-L-lysine. Cells were incubated at 37° in a humidified incubator with 5% CO_2 and 95% air. After reaching confluency (approximately 7 days), cells were dissociated from the dishes with trypsin (0.25%), washed with DMEM/CBSd, and centrifuged at 1000 × *g* for 10 min. Cells were resuspended at a density of 1 × 10^6/ml in DMEM/CBSd, and 2 ml were plated in 35-mm dishes. Cells were allowed to grow to confluency (another 4–5 days) before experimentation. Total age of the cells (after removal from brain) was typically between 12 and 21 days at the time experiments were performed.

[^{3}H]InsP accumulation in cultured cells. NE-stimulated accumulation of [^{3}H]InsPs was determined in primary glial cultures, as previously described (17). Briefly, plates were prelabeled for 10 days with [^{3}H]inositol (1 μCi/2 ml), medium was aspirated, plates were washed with 2 ml of KRB, and 1 ml of KRB was added. Some cultures were pretreated with 100 μM CEC for 30 min in KRB and washed three times with 1 ml of KRB. Cultures were incubated with the indicated concentrations of NE, in 1 ml of KRB containing 10 mM LiCl (only 110 mM NaCl), for 1 hr. Reactions were stopped with methanol, and [^{3}H]InsPs were isolated by anion exchange chromatography, as described previously (17).

Data analysis. EC_{50} values were calculated by linear regression of all points between 20% and 80% of the maximal response. Where appropriate, K_I values were calculated from IC_{50} values by the method of Cheng and Prusoff (18).

Results

Selective alkylation of α_1- and β-adrenergic receptor-mediated responses in slices. Because α_1-selective agonists have a low intrinsic activity for activating second messenger responses in brain slices, NE is the best agonist for activating these receptors. However, NE stimulates all adrenergic receptor subtypes, and it is necessary to determine the contributions of different subtypes to each response. As previously (14), we used selective alkylating agents to eliminate contributions of particular subtypes. PBZ was used to inactivate α_1-adrenergic receptors (19), BIM to inactivate β-adrenergic receptors (20), and/or CEC to selectively inactivate the α_{1B} subtype (3).

Table 1 shows that PBZ pretreatment of cortical slices dose-dependently blocked the NE-mediated potentiation of cAMP accumulation in response to both isoproterenol or adenosine, consistent with mediation by an α_1 receptor. The concentration dependence for PBZ inactivation of this response was almost identical to that reported previously for inactivation of α_1-mediated InsP formation in the same slice preparation (14). Concentrations of PBZ sufficient to completely inactivate the α_1 component did not substantially alter the response to either β-adrenergic or adenosine receptor activation (Table 1).

Pretreatment with BIM completely eliminated the response to β-adrenergic receptor activation, without affecting the response to adenosine or the α_1-mediated potentiation of the adenosine response (Table 1). Similarly, a small response to NE was still seen, consistent with the small α_1-mediated increase in basal cAMP accumulation reported previously (14).

Pretreatment with a combination of BIM and PBZ eliminated all responses to both β and α_1 activation, without substantially affecting responses to adenosine (Table 1), as expected. Conversely, treatment with a combination of BIM and CEC eliminated the β response, without affecting either the response to adenosine or the α_1-mediated potentiation of this response (Table 1), consistent with our previous observation that this α_1 potentiation is insensitive to CEC (21).

In all subsequent experiments, α_1-mediated potentiation of adenosine-mediated cAMP potentiation was examined in BIM-pretreated slices, to eliminate confusion caused by β-adrenergic receptor activation.

[^{3}H]InsP and [^{3}H]cAMP in slices with prazosin, phentolamine, and yohimbine. Inhibition of NE-stimulated InsP formation and NE-mediated potentiation of cAMP responses to isoproterenol and adenosine by the α_1-selective antagonist

TABLE 1

Agonist-stimulated cAMP accumulation in cortical slices after pretreatment with selective alkylating agents

[^{3}H]Adenine-prelabeled cortical slices were pretreated with the indicated alkylating agents, as described in the text. After extensive washing, cAMP accumulation in response to isoproterenol (ISO) (10 μM), NE (100 μM), adenosine (ADEN) (100 μM), or the indicated combinations was determined as described. Values are expressed as percentage of the response to isoproterenol or adenosine in control slices pretreated without alkylating agents. Each value is the mean ± standard error from three to six experiments.

	ISO response			ADEN response		
	ISO	NE	ISO + NE	ADEN	ADEN + NE	ADEN + ISO
	% of control			% of control		
Control	100	233 ± 36	278 ± 36	100	369 ± 50	157 ± 30
0.01 μM PBZ	95 ± 3	156 ± 13	170 ± 43	85 ± 12	329 ± 64	171 ± 19
1 μM PBZ	80 ± 8	65 ± 28	80 ± 15	84 ± 6	117 ± 15	120 ± 8
10 μM BIM	−2 ± 14	32 ± 2	40 ± 14	134 ± 14	370 ± 22	161 ± 8
BIM + 1 μM PBZ	−2 ± 5	0 ± 7	2 ± 5	69 ± 4	97 ± 11	89 ± 9
BIM + 100 μM CEC	−22 ± 8	−17 ± 6	22 ± 19	115 ± 6	315 ± 21	154 ± 13

prazosin, the α_2-selective antagonist yohimbine, and the broad spectrum α-antagonist phentolamine were determined in brain slices. For each response, the order of potency of the antagonists was prazosin > phentolamine > yohimbine, as expected at an α_1-adrenergic receptor. However, the potencies of each antagonist varied substantially between tissues (Table 2). In particular, prazosin was 14–54-fold less potent in blocking cAMP responses than in blocking the InsP response.

[^{3}H]InsP and [^{3}H]cAMP in slices with (+)-niguldipine and 5-MU. The effects of the subtype-selective antagonists (+)-niguldipine and 5-MU on InsP and cAMP responses to α_1 receptor activation in cortical slices are shown in Fig. 1. As reported previously (12), (+)-niguldipine did not substantially inhibit the α_1 potentiation of cAMP responses to either isoproterenol or adenosine, even at concentrations up to 10 μM. Conversely, NE-stimulated InsP formation in slices of both neocortex and hippocampus was blocked with an IC_{50} around 100 nM. 5-MU blocked all three responses, with IC_{50} values ranging from 23 to 704 nM (Table 2).

The competitive or noncompetitive nature of inhibition by each of these compounds was studied by performing concentration-response curves to NE in the presence of fixed concentrations of each antagonist (Figs. 2 and 3). Both (+)-niguldipine and 5-MU appeared to be competitive antagonists of NE-stimulated InsP formation (Figs. 2 and 3, *top*). Schild plots of these data (22) resulted in pA_2 values of 7.8 and 8.2 for (+)-niguldipine and 5-MU, respectively, in inhibiting the InsP response (slopes of 0.6 and 1.2, respectively; data not shown). (+)-Niguldipine (3 μM) had little effect on NE-mediated potentiation of cAMP accumulation, as expected (Fig. 2). The inhibition of NE-mediated potentiation of cAMP accumulation by 5-MU was, however, generally noncompetitive, in the presence of either isoproterenol or adenosine (Fig. 3).

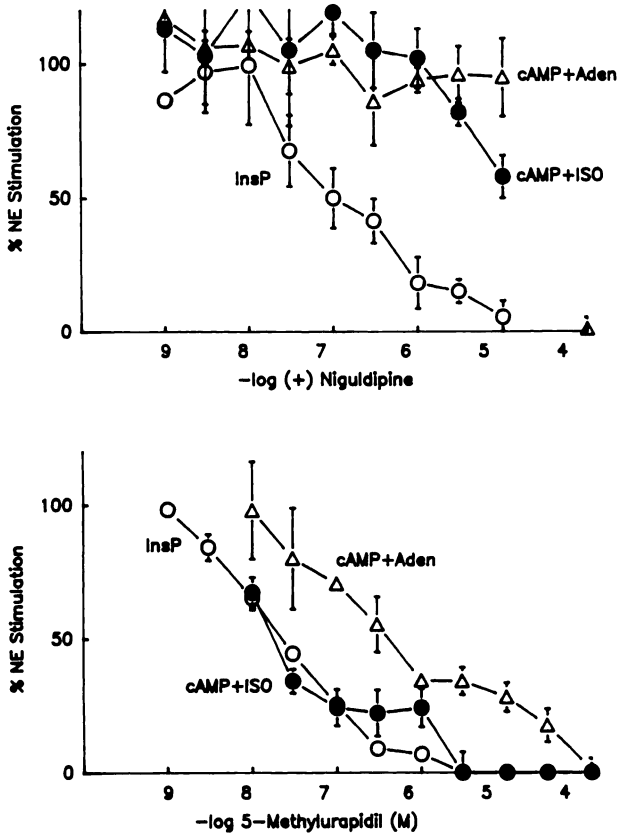

Fig. 1. Effect of (+)-niguldipine and 5-MU on NE-stimulated second messenger responses in neocortical slices. Inhibition of NE (100 μM)-stimulated [^{3}H]InsP formation or NE (100 μM)-induced potentiation of the cAMP response to isoproterenol (*ISO*) (10 μM) or adenosine (*Aden*) (100 μM) was determined in slices of neocortex using different concentrations of (+)-niguldipine (*top*) or 5-MU (*bottom*). [^{3}H]cAMP accumulation in the presence of adenosine was performed in BIM (10 μM, 10 min)-pretreated slices. Each value is the mean ± standard error of three to five experiments, each performed in duplicate or triplicate.

The effect of (+)-niguldipine (3 μM) on InsP and cAMP responses to a variety of different agonists in brain slices was also determined. (+)-Niguldipine blocked InsP responses to NE, epinephrine, 6-fluoro-NE, α-methyl-NE, phenylephrine, and methoxamine (all at 100 μM), regardless of their intrinsic activity. However, this compound did not significantly inhibit

TABLE 2

Potencies of antagonists against NE-stimulated cAMP and InsP formation in slices of rat neocortex and hippocampus

Dose-response curves for inhibition of the response to NE (100 μM) were determined as described in the text. InsP responses were examined in cerebral cortex and hippocampus and cAMP responses in cerebral cortex in the presence of 10 μM isoproterenol (+ISO) or 100 μM adenosine (+Aden). cAMP responses to NE plus adenosine were measured in BIM-pretreated (10 μM, 10 min) slices. Each value is the mean ± standard error from three or four experiments.

	IC_{50}			
	Cortex InsPs	Hippocampus InsPs	Cortex cAMP (+ISO)	Cortex cAMP (+Aden)
	nM			
Prazosin	18 ± 0.3	ND*	254 ± 78	980 ± 514
Phentolamine	610 ± 20	ND	700 ± 100	2,640 ± 1,250
Yohimbine	9,825 ± 2,799	ND	1,894 ± 201	14,840 ± 6,970
(+)-Niguldipine	112 ± 42	92 ± 32.5	>10,000	>10,000
5-MU	23 ± 3.8	33 ± 5.5	24 ± 3.2	704 ± 109

* ND, not determined.

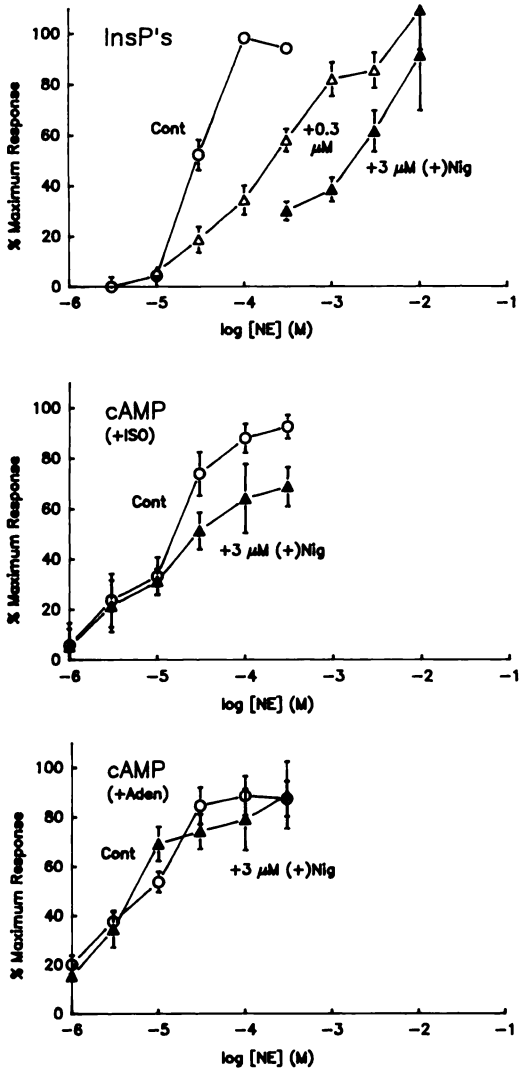

Fig. 2. Effect of (+)-niguldipine [*(+)Nig*] on concentration-response curves for NE-stimulated [^{3}H]InsP formation (*top*) or potentiation of the cAMP response to isoproterenol (*ISO*) (10 μM) (*middle*) or adenosine (*Aden*) (100 μM) (*bottom*). [^{3}H]cAMP accumulation in the presence of adenosine was performed in BIM (10 μM, 10 min)-pretreated slices. Each value is the mean ± standard error of three or four experiments, each performed in duplicate or triplicate. *Cont*, control.

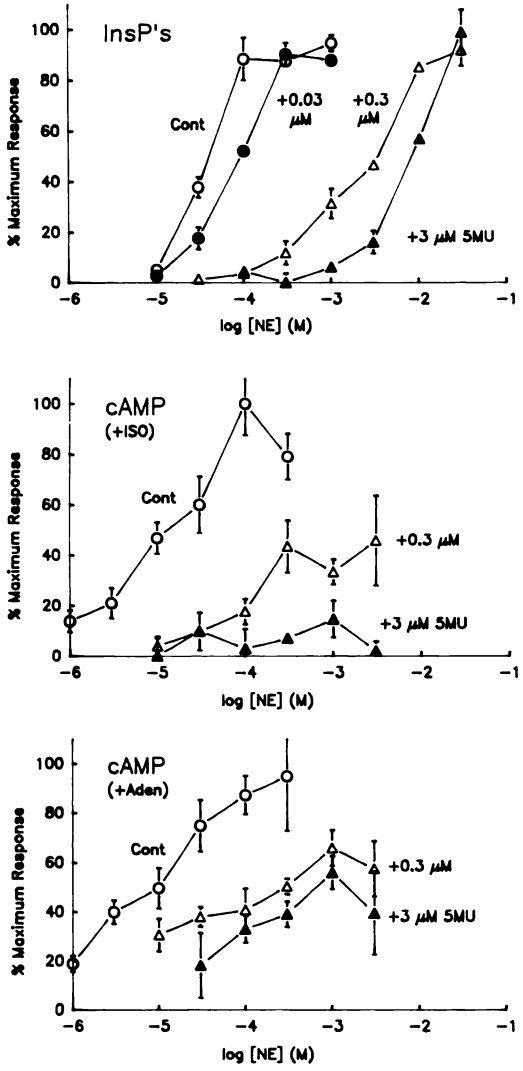

Fig. 3. Effect of 5-MU on concentration-response curves for NE-stimulated [^{3}H]InsP formation (*top*) or potentiation of the cAMP response to isoproterenol (*ISO*) (10 μM) (*middle*) or adenosine (*Aden*) (100 μM) (*bottom*). [^{3}H]cAMP accumulation in the presence of adenosine was performed in BIM (10 μM, 10 min)-pretreated slices. Each value is the mean ± standard error of three or four experiments, each performed in duplicate or triplicate.

cAMP responses to any of these agonists, in the presence of either isoproterenol or adenosine (data not shown).

[^{3}H]InsPs in primary glial cultures with (+)-niguldipine, 5-MU, and WB 4101. We have previously reported that [^{3}H]InsP responses to NE in primary cultures of glial cells from neonatal rat brain are not substantially blocked by CEC pretreatment (17), suggesting that they are mediated primarily by the α_{1A} subtype. It was surprising, therefore, to find that (+)-niguldipine exhibited a relatively low potency (~100 nM) (Fig. 4) in blocking this response, more consistent with involvement of the α_{1B} subtype. Pretreatment of cultures with CEC to inactivate the α_{1B} subtype would be expected to increase the proportion of α_{1A} involvement in this response and increase the potency of (+)-niguldipine; however, this did not occur (Fig. 4). Instead, a slight decrease in the potency of (+)-niguldipine was observed after CEC pretreatment.

The pharmacological properties of the receptors mediating this response were further examined by comparing the potencies of subtype-selective antagonists in inhibiting responses to different concentrations of NE (Fig. 5). WB 4101 and 5-MU were very potent in blocking InsP responses to NE in primary glial cultures. This inhibition was competitive, because substantially higher concentrations of antagonist were necessary to block responses to higher agonist concentrations. However, (+)-niguldipine showed essentially noncompetitive inhibition of the NE response, being equally potent in blocking responses to concentrations of NE varying by 100-fold (Fig. 5).

After CEC pretreatment of cultures, similar patterns were observed (data not shown). Both WB 4101 and 5-MU retained a high potency and showed competitive behavior. Although (+)-niguldipine began to show some aspects of competitive interaction, the interaction was still not completely competitive, because increasing the NE concentration by 100-fold caused only a 10-fold change in the potency of (+)-niguldipine (Table 3).

Further analysis of the inhibition by (+)-niguldipine is shown

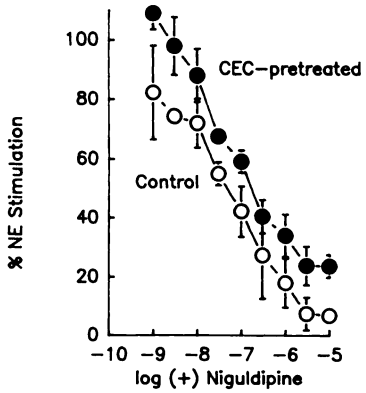

Fig. 4. Inhibition of NE-stimulated [^{3}H]InsP formation in primary glial cultures by (+)-niguldipine. [^{3}H]Inositol-prelabeled cultures were pretreated with (●) or without (○) CEC (100 μM, 30 min). NE (100 μM)-stimulated [^{3}H]InsP formation was determined at the indicated concentrations of (+)-niguldipine. Values are presented as a percentage of the response to NE in the absence of antagonist in either control or CEC-pretreated cultures. Each value is the mean ± standard error of four to six experiments performed in duplicate.

in Fig. 6, where concentration-response curves for NE in activating InsP formation in primary glial cultures were examined in the presence of fixed (+)-niguldipine concentrations. In control cultures the inhibition by (+)-niguldipine was essentially noncompetitive, whereas in CEC-pretreated cultures a mixed competitive/noncompetitive inhibition was observed.

IC_{50} and K_I values for each antagonist in these cultures are listed in Table 3. Both WB 4101 and 5-MU gave calculated K_I values that were essentially independent of agonist concentration, as expected for competitive inhibition. The K_I values in control cells resembled those expected at the α_{1A} subtype for these antagonists. Surprisingly, CEC pretreatment caused a small but significant increase in the average K_I value for both WB 4101 and 5-MU (Table 3). The noncompetitive nature of the inhibition by (+)-niguldipine precluded calculation of K_I values; however, the potency of this compound resembled that expected at the α_{1B} subtype.

Lack of effect of nifedipine. To ensure that the Ca^{2+} channel-blocking properties of (+)-niguldipine (a dihydropyridine) were not confusing the results, the effect of nifedipine, a Ca^{2+} channel-blocking dihydropyridine with no α_1 antagonist activity, was examined in each preparation. Nifedipine (0.01–10 μM) had no effect on NE-stimulated InsP formation or potentiation of cAMP accumulation in brain slices or on NE-stimulated InsP formation in primary glial cultures (data not shown). Moreover, addition of nifedipine (10 μM) did not alter the potency of (+)-niguldipine in blocking any of these responses (data not shown).

Discussion

The existence of different second messenger responses to α_1-adrenergic receptor activation in brain slices was an important factor leading to the identification of pharmacologically distinct subtypes of this receptor (5, 21). Based on these and other studies, alkylating agents and competitive antagonists were identified that differentiated between α_1 receptors in different tissues (1–5) and led to the currently accepted pharmacological definition of α_{1A} and α_{1B} subtypes (5). Functional studies suggest that the α_{1A} subtype is primarily linked to Ca^{2+} influx, whereas the α_{1B} subtype activates InsP formation and Ca^{2+}

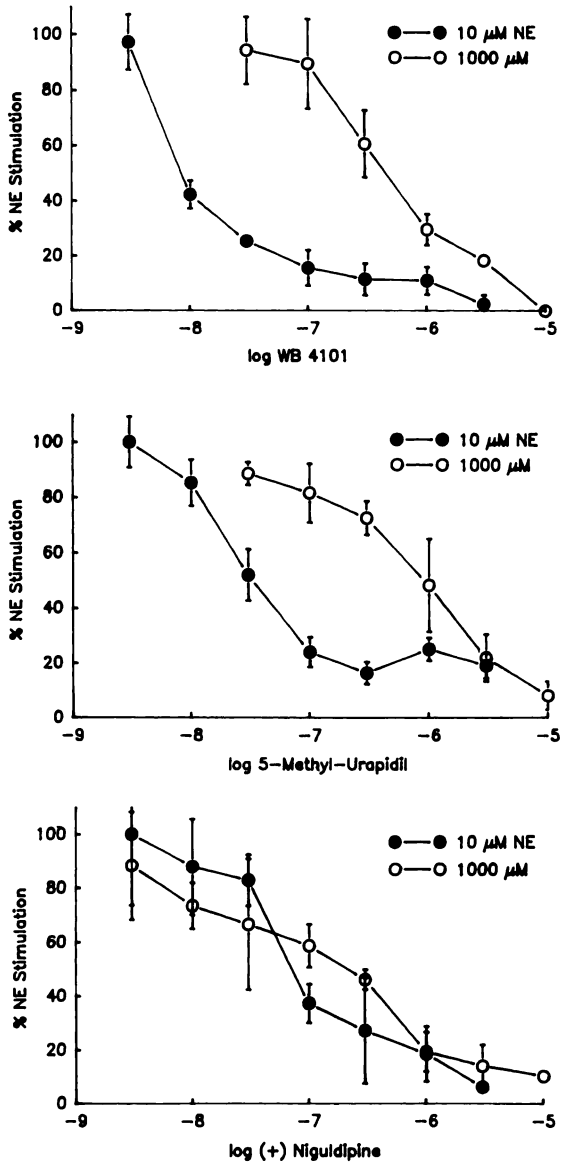

Fig. 5. Inhibition of NE-stimulated [^{3}H]InsP formation in primary glial cultures by subtype-selective antagonists. The indicated concentrations of WB 4101 (*top*), 5-MU (*middle*), or (+)-niguldipine (*bottom*) were examined for [^{3}H]InsP responses to 10 μM NE (●) or 1000 μM NE (○). Values are presented as a percentage of the response to NE in the absence of antagonist. Each value is the mean ± standard error of three or four experiments performed in duplicate.

mobilization (2, 6, 9, 24, 25). These conclusions have been drawn primarily from experiments utilizing CEC and WB 4101 to differentiate these subtypes.

However, the relationship of the α_{1A} and α_{1B} subtypes to the different second messenger responses in brain slices has remained unclear (23). This has been due mainly to the relatively low selectivity of available competitive antagonists (WB 4101 and phentolamine) and uncertainties about the penetration of CEC into tissue slices (5). The recent introduction of (+)-niguldipine and 5-MU, competitive antagonists with α_{1A}/α_{1B} selectivity ratios of 100 or greater (8–11), would be expected to permit a clearer definition of the receptor subtypes involved in such responses. We, therefore, utilized these new drugs to reexamine second messenger responses in brain slices and cultures.

TABLE 3

Potencies of subtype-selective antagonists against NE-stimulated [^{3}H]InsP formation in control or CEC-pretreated glial cultures

Confluent glial cultures were prelabeled with [^{3}H]inositol for 7–12 days. Stimulation of [^{3}H]InsP formation by the indicated concentrations of NE was determined in the absence or presence of different concentrations of each antagonist in control cultures or cultures pretreated with 100 μM CEC for 30 min. IC_{50} and K_I values were calculated as described in the text. Each value is the mean ± standard error of three to five determinations.

Control cultures	IC_{50}			Calculated K_I			Average K_I
	10 μM NE	100 μM NE	1000 μM NE	10 μM NE	100 μM NE	1000 μM NE	
		nM			nM		nM
WB 4101	9.4 ± 5.4	116 ± 4	282 ± 42	1.6 ± 0.9	2.3 ± 0.1	0.6 ± 0.01	1.5 ± 0.5
5-MU	28 ± 3.8	310 ± 30	913 ± 385	4.7 ± 0.6	6.1 ± 0.6	1.8 ± 0.8	4.2 ± 1.3
(+)-Niguldipine	62 ± 12	72 ± 25	176 ± 73	NC[a]	NC	NC	NC
CEC-pretreated cultures	IC_{50}			Calculated K_I			Average K_I
	30 μM NE	100 μM NE	3000 μM NE	30 μM NE	100 μM NE	3000 μM NE	
		nM			nM		nM
WB 4101	24 ± 4.4	62 ± 18	796 ± 248	6.0 ± 1.1	5.6 ± 1.6	2.6 ± 0.8	4.7 ± 1.1
5-MU	61 ± 4.6	92 ± 36	2,855 ± 787	15.0 ± 1.2	8.4 ± 3.3	9.5 ± 2.6	10.9 ± 3.5
(+)-Niguldipine	71 ± 11.5	144 ± 42	762 ± 163	NC	NC	NC	NC

[a] NC, not calculated, because the noncompetitive nature of the inhibition invalidates the formula used for calculation.

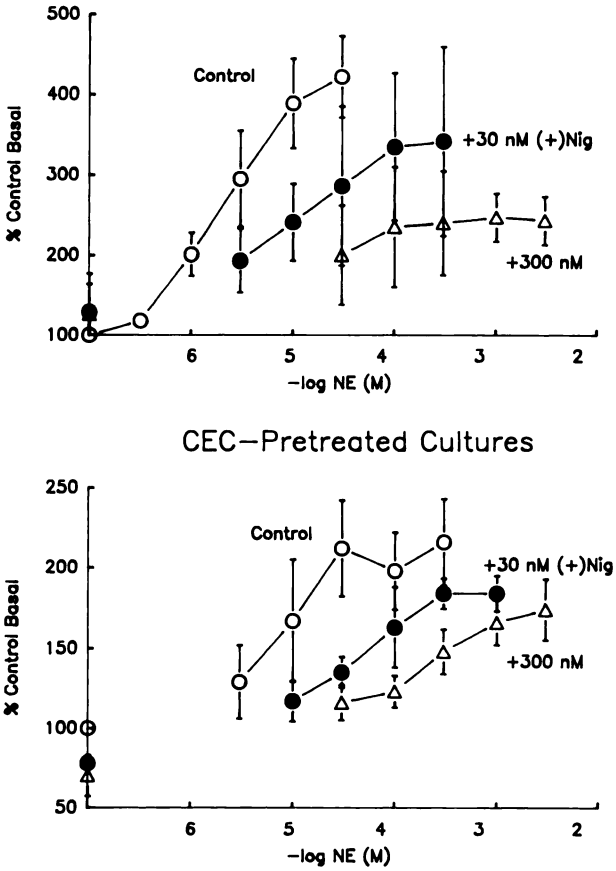

Fig. 6. Concentration-response curves for NE-stimulated [^{3}H]InsP formation in control (*top*) and CEC-pretreated (*bottom*) primary glial cultures in the absence (○) or presence of 30 nM (●) or 300 nM (△) (+)-niguldipine. Values are presented as percentage of basal values in the absence of agonist. Each value is the mean ± standard error of four experiments performed in duplicate.

InsP formation in brain slices. Evidence now suggests that both the α_{1B} (2, 4–6) and α_{1A} (6, 17, 26, 27) subtypes can activate InsP formation in different tissues, although different mechanisms seem to be involved in responses to each subtype (6, 7, 26, 27). Michel *et al.* (28) recently reported that (+)-niguldipine had a relatively low potency in blocking NE-stimulated InsP formation in cortical slices, concluding that this response is solely α_{1B}. Although Robinson and Kendall (12) found similar results with (+)-niguldipine, they found that (−)-niguldipine was even less potent (100-fold). In radioligand binding studies, only the α_{1A} subtype shows stereoselectivity for niguldipine enantiomers (10, 11). In addition, Johnson and Minneman (21) showed previously that this response is not blocked by CEC pretreatment. In this manuscript, we report that 5-MU has K_I values of 4–6 nM (correcting for NE saturation) in blocking this response in slices from both cerebral cortex and hippocampus (enriched in the α_{1A} subtype). This is most similar to K_D values for 5-MU at α_{1A} binding sites and is about 25 times lower than the K_D for the α_{1B} subtype (8, 9, 29).

Thus, the α_1 receptor linked to InsP formation in brain slices appears to have mixed characteristics. Although its relatively low affinity for (+)-niguldipine suggests that it is α_{1B}-like, its stereoselectivity for niguldipine enantiomers, insensitivity to CEC, and relatively high affinity for 5-MU suggest that it is α_{1A}-like.

Potentiation of cAMP accumulation in brain slices. Robinson and Kendall (12) first reported that (+)-niguldipine showed only a very small inhibition of NE-mediated potentiation of isoproterenol-induced cAMP accumulation in cortical slices, even at high concentrations. In conjunction with prior pharmacological data (14, 21, 23), it seems probable that a novel α_1 receptor subtype is involved in this response.

Here we confirm the fact that (+)-niguldipine is essentially inactive in blocking the NE-mediated potentiation of the cAMP response to isoproterenol and show that it is also unable to block the much larger NE-mediated potentiation of the cAMP response to adenosine. These responses are clearly of the α_1 subtype, because antagonists block them with an order of potency of prazosin > phentolamine > yohimbine and they are highly sensitive to inactivation by PBZ pretreatment. The lack of effect of (+)-niguldipine was observed with a variety of catecholamine and phenylethylamine agonists and was apparent at all concentrations of NE examined. 5-MU was able to block both types of potentiative responses with potencies not unlike those at the α_{1A} and α_{1B} subtypes. However, this inhibition appeared to be primarily noncompetitive, precluding the use of K_I values for this antagonist in classifying these receptors.

Overall, it is clear that the α_1 receptors potentiating cAMP

accumulation in response to isoproterenol and adenosine in cortical slices also do not fit with the current α_{1A}/α_{1B} subclassification scheme. In addition, they are clearly different from the receptors linked to InsP formation in the same slice preparation. The extremely low potency of (+)-niguldipine for these receptors should be useful for attempting to identify similar receptors in other tissues and identifying the receptor binding sites involved in these responses.

InsP formation in primary glial cultures. NE-stimulated InsP formation in primary glial cultures is clearly due to α_1-adrenergic receptor activation, because prazosin is 40–100-fold more potent than yohimbine in blocking this response (7, 30, 31). These cultures contain both α_{1A} and α_{1B} binding sites, as defined by WB 4101 inhibition curves and CEC inactivation (7). Inactivation of the α_{1B} receptors by pretreatment with CEC only slightly decreased the InsP response to NE, although the potency of NE was decreased 9-fold (7). The insensitivity of this response to CEC led us to conclude previously that it was mediated primarily by the α_{1A} subtype (7).

Here we show that the subtype-selective antagonists WB 4101 and 5-MU competitively antagonize NE-stimulated InsP formation in primary glial cultures and that the calculated K_I values for these compounds closely resemble their K_D values in binding to the α_{1A} subtype. This is consistent with the insensitivity of this response to CEC (7). Again, however, (+)-niguldipine behaves anomalously. (+)-Niguldipine shows a relatively low potency in blocking this response (IC_{50} around 100 nM). Although this is reminiscent of its K_D in binding to the α_{1B} subtype, the noncompetitive nature of this inhibition precludes calculation of a K_I value. It is clear, however, that concentrations of (+)-niguldipine that should occupy >95% of the α_{1A} subtype (10–30 nM) do not significantly inhibit the response to even a low concentration of NE (see Fig. 5). This is inconsistent with mediation of this response by the α_{1A} subtype.

Pretreatment with CEC to inactivate the α_{1B} subpopulation should increase the α_{1A}-like nature of this response and might be expected to increase the potency of α_{1A}-selective antagonists. This was not observed, and CEC pretreatment actually caused a small decrease in the potency of WB 4101 and 5-MU. CEC pretreatment also decreased the apparent potency of (+)-niguldipine, although this inhibition remained substantially noncompetitive.

Thus, the receptors mediating the InsP response to NE in primary glial cultures also do not fit easily into the α_{1A}/α_{1B} classification scheme. Their general insensitivity to CEC and high affinity for 5-MU and WB 4101 suggest α_{1A}-like properties, but their relatively low affinity for (+)-niguldipine and the noncompetitive nature of this inhibition are not consistent with interaction with the α_{1A} subtype.

Interactions between subtypes? It is possible that both α_{1A} and α_{1B} subtypes interact to influence InsP formation. Both subtypes might activate InsP formation in a redundant manner, where blockade or removal of either subtype alone will not affect the response. Conversely, the response might require synergistic activation of both subtypes, where blockade or removal of either subtype alone will eliminate the response. However, neither of these mechanisms would explain our results. With redundant activation where both subtypes must be blocked to inhibit the response, the potencies of selective drugs would reflect that subtype at which they are least potent. Conversely, with synergistic activation where blocking of either subtype will inhibit the response, the potencies of selective drugs will reflect that subtype at which they are most potent. We found that in both brain slices and glial cultures the potency of (+)-niguldipine was similar to that at the subtype at which it is least potent (α_{1B}), whereas the potency of 5-MU was similar to that at the subtype at which it is most potent (α_{1A}).

Additional subtypes? These studies clearly raise more questions than they answer. However, several general conclusions can be drawn. Most interesting is the fact that none of the second messenger responses studied are blocked by (+)-niguldipine with the high affinity (1 nM or less) found at the α_{1A} subtype in radioligand binding studies. Thus, this subtype has not yet been clearly linked to a second messenger system. This fact is particularly intriguing in light of the increasing evidence linking the α_{1A} subtype to Ca^{2+} influx (2, 6, 9, 24, 25).

It also seems likely that there is a subtype of α_1-adrenergic receptor with a very low affinity (>3 μM) for (+)-niguldipine that has not yet been revealed by radioligand binding studies. This would be the receptor involved in potentiating the cAMP responses to other G_S-linked receptors.

Finally, it is clear that the current α_{1A}/α_{1B} subclassification scheme is inadequate to explain any of the second messenger responses studied here. (+)-Niguldipine, 5-MU, and WB 4101 are all α_{1A}-selective in radioligand binding studies, with approximately similar K_D values (1 nM) at the α_{1A} subtype. However, the potencies of these compounds do not co-vary at the different second messenger responses. For example, (+)-niguldipine is about 5-fold more potent than 5-MU in blocking InsP responses to high concentrations of NE in glial cultures but is 5-fold less potent in blocking the same response in cortical slices and about 400-fold less potent in blocking the cAMP response in cortical slices. In addition, some noncompetitive inhibition by both (+)-niguldipine and 5-MU was observed at different responses. These data suggest that there may be additional subtypes of α_1-adrenergic receptors and that careful pharmacological analysis with several subtype-selective antagonists will be necessary to make accurate conclusions about which subtypes of α_1-adrenergic receptors mediate particular functional responses.

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