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EFFECTS OF CLONIDINE AND OTHER IMIDAZOLE-RECEPTOR BINDING AGENTS ON SECOND MESSENGER SYSTEMS AND CALCIUM INFLUX IN BOVINE ADRENAL CHROMAFFIN CELLS

SOUNDARARAJAN REGUNATHAN,* MARIAN J. EVINGER, MARY P. MEELEY and DONALD J. REIS

Division of Neurobiology, Department of Neurology and Neuroscience, Cornell University Medical College, New York, NY 10021, U.S.A.

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Abstract—Clonidine and related imidazoline compounds bind to α_2-adrenergic as well as to newly described non-adrenergic imidazole/imidazoline receptors in brain and peripheral tissues. The present study was undertaken to identify the signal transduction mechanism coupled to this new class of receptors (imidazole receptors) using bovine adrenal chromaffin cells. Clonidine did not modify the basal or forskolin-stimulated production of cyclic AMP (cAMP), suggesting the absence of functionally active α_2-adrenergic receptors in adrenal chromaffin cells. Clonidine also failed to modify the basal and GTPγS- or carbachol-stimulated increase in phosphoinositide hydrolysis. However, clonidine increased significantly the production of cyclic GMP (cGMP) as well as the uptake of $^{45}Ca^{2+}$. The cGMP response to clonidine was slower (peak at 15 min) and smaller (only about 50% over control) than the response to acetylcholine and was not shared by other agents that bind to imidazole receptors. In contrast, all agents that bind to imidazole receptors increased the influx of $^{45}Ca^{2+}$ into chromaffin cells. It is concluded that (a) α_2-adrenergic and imidazole receptors are functionally distinct and linked to different signal transduction mechanisms; (b) the classical G-protein coupled soluble second messenger systems are not coupled to imidazole receptors; (c) clonidine may increase cGMP by a non-receptor-mediated intracellular action; and (d) imidazole receptors may regulate intracellular calcium levels through an ion regulating system that may be different from calcium channels.

Clonidine, a phenylimidazoline, has traditionally been viewed as an α_2-adrenergic partial agonist [1]. However, there is increasing evidence that clonidine also interacts in brain and other organs with a novel class of receptors which bind imidazol(in)es as well as some oxazoles (e.g. rilmenidine [2, 3]) and compounds with a guanidinium moiety with high affinity [4]. This class of receptors has variously been termed the imidazole receptor (IR) [5], the imidazoline-preferring receptor (IPR) [6] or the imidazoline–guanidinium receptor [4]. That the IR is distinct from the α_2-adrenergic receptor has been supported by the facts that the two receptors are unequally distributed within brain and kidney [4, 7], that the purported protein structure of these two receptors appears to be different [8], and that transfection of cell lines with genes for the α_2-adrenergic receptor does not confer IR binding properties on those cells [9]. While α_2-adrenergic receptors are primarily coupled through an inhibitory G-protein to inhibit cyclic AMP (cAMP) production [10], the signal transduction mechanism associated with the IR is not known.

One tissue that expresses IRs with no apparent co-expression of α_2-adrenergic receptors is the adrenal medulla, specifically chromaffin cells [11]. Clonidine and other imidazole agents bound only to IRs in bovine chromaffin cells, with no α_2-adrenergic binding (unpublished observation). Clonidine also inhibits the release of catecholamines induced by cholinergic agonists, an effect that is not reversed by α_2-antagonists [12]. Thus, the binding and action of clonidine appear to occur at the IR in these cells.

We have reported recently that in whole rat adrenal gland clonidine does not modify phosphoinositide (PI) turnover or production of cAMP. However, it elicits a slow and modest increase in the production of cyclic GMP (cGMP) [13]. These findings raise the possibility that a transduction mechanism for IRs, at least in the whole adrenal gland, differs from that associated with activation of the α_2-adrenergic receptor. However, whether the effects of clonidine in adrenal gland are attributable entirely to interaction with chromaffin cells, and whether effects upon cGMP production are a general feature of occupancy of cell-surface IRs have yet to be ascertained.

In the present investigation we have, therefore, sought to determine the signal transduction mechanisms coupled to IR in primary cultures of chromaffin cells of the bovine adrenal medulla. This has been accomplished by comparing the effects of clonidine with other agents which bind to IR on the accumulation of cAMP and cGMP, on PI turnover,

* Correspondence: S. Regunathan, Ph.D., Division of Neurobiology, Cornell University Medical College, 411 East 69th St., New York, NY 10021, U.S.A.

and on the intracellular accumulation of calcium in these cells. Our results indicate that IRs are functionally different from α_2-adrenergic receptors and that the occupancy of IR is associated with an increased accumulation of intracellular Ca^{2+} in adrenal chromaffin cells. A preliminary report of parts of this study has been presented in abstract [14].

EXPERIMENTAL PROCEDURES

Primary culture of adrenal chromaffin cells

Monolayer primary cultures of chromaffin cells were prepared from bovine adrenal glands by the method of Wilson and Viveros [15], as modified by Ross *et al.* [16].

Bovine adrenal glands were obtained from a local slaughterhouse within 4 hr post-mortem. The glands were perfused with collagenase (2 mg/mL) (Worthington) and DNAse (50 μg/mL) (Sigma Chemical Co.), and medulla was then dissected from cortex. The minced tissue was further digested in collagenase for 40–60 min and filtered through a 105 μm wire sieve. The resulting cell suspension was loaded onto a step gradient of 15% and 7.5% Renografin (Squibb) and centrifuged for 20 min at 10,000 *g*. Chromaffin cells were collected from the interface of the gradients, washed and plated at required density in a Dulbecco's Modified Eagle's Medium (DMEM) : F12 nutrient mixture (1 : 1) supplemented with 10 mM HEPES buffer (pH 7.4), 10% fetal bovine serum and 1% antibiotics (penicillin and streptomycin). The cells were used for experiments between days 3 and 5 of culture.

Measurement of PI turnover

PI turnover was measured by estimating the accumulation of inositol-1-phosphate in the presence of lithium in chromaffin cells in culture according to the method of Berridge *et al.* [17].

Cells (1×10^6; 24-well plate) were prelabeled with 2 μCi [^{3}H]inositol for about 16 hr in cell culture medium. Following labeling, they were washed three times with 1 mL of Krebs–Ringer bicarbonate (KRB) buffer containing 10 mM unlabeled inositol. To initiate PI hydrolysis, cells were incubated, in the presence of drugs, for 30 min in KRB containing 10 mM lithium chloride in a total volume of 0.5 mL. The reaction was stopped by the addition of methanol (0.5 mL), and cells were collected into tubes. The wells were washed again with another 0.5 mL methanol and the washings combined. To the tubes containing the harvested cells, 0.5 mL of distilled water and 0.5 mL of chloroform were added and the mixture was sonicated. After mixing, the tubes were then centrifuged at 500 *g* for 10 min to separate aqueous and lipid phases, and an aliquot of the lipid phase was removed for counting of radioactivity. The aqueous phase was passed through an AG 1 × 8 (Bio-Rad) column. Free inositol, glycerophosphates and cyclic phosphates were eluted using 15 mL of 5% inositol and 2 mL of 60 mM sodium formate/5 mM sodium borate, respectively. Inositol-1-phosphate was eluted using 5 mL of 0.2 M ammonium formate in 0.1 M formic acid, and collected into scintillation vials; then the radioactivity was measured. Results represent dpm of inositol-1-phosphate expressed as percent of total dpm (inositol-1-phosphate + lipid dpm).

Production of cAMP and cGMP

The amount of cyclic nucleotide produced in the presence of drugs was measured by radioimmunoassay after extraction of incubated cells with 70% ethanol [18]. To compare the effect of clonidine in adrenal chromaffin cells with a well-established α_2-adrenergic receptor-mediated response, the production of cAMP in rat brain was also measured. For this purpose, miniprisms (350 μm) of cerebral cortex were prepared using a McIlwain tissue chopper. Chromaffin cells (1×10^6 cells; 24-well plates)/cortical miniprisms (in test tubes) were first washed and then preincubated at 37° for 30 min in KRB. Drugs were added to start the incubation with a final volume of 0.5 mL. At the end of the incubation period, 0.5 mL of ethanol was added to stop the reaction. The cells were harvested from each well into individual tubes, the wells were washed with another 0.5 mL of ethanol, and the washings were combined. The cells and the cortical miniprisms were sonicated and left on ice for 10 min. Precipitated proteins were separated by centrifugation, the precipitates were washed once with 0.5 mL ethanol, and the supernatants were combined. The final ethanol extract was evaporated under vacuum (Speed-Vac; Savant Instruments) and the residue dissolved in an appropriate volume of assay buffer. Assays for cAMP and cGMP were carried out using the non-acetylated protocols with the Amersham radioimmunoassay kits. The protein content was assayed by the Coomassie blue method [19].

Uptake of $^{45}Ca^{2+}$ by chromaffin cells

The cells (2×10^6 cells; 6-well plates) were washed with Krebs–Ringer solution containing 25 mM HEPES (pH 7.4) and preincubated for 1 min in the same buffer containing 1 μCi $^{45}Ca^{2+}$. Each drug indicated was added to start the incubation and the uptake was terminated by rapid aspiration of the medium. The cells were washed four times with ice-cold, calcium-free buffer, then solubilized using 1% Triton X-100 and collected into scintillation vials. The radioactivity was counted in a liquid scintillation system having 90% efficiency for $^{45}Ca^{2+}$. The amount of calcium taken up by the cells was calculated from the initial concentration of calcium in the medium (2.5 mM) and the results are expressed as nmol calcium/10^6 cells.

Uptake of [^{3}H]clonidine by chromaffin cells

Chromaffin cells (2×10^6 cells; 6-well plates) were washed with warm Krebs–HEPES buffer (pH 7.4) and the uptake incubation was started by the addition of [^{3}H]clonidine with various concentrations of unlabeled clonidine in Krebs–HEPES buffer. The cells were incubated for 10 min at 37° in a final volume of 1 mL. Uptake was stopped by rapid aspiration of medium and cells were washed three times with ice-cold buffer, solubilized in 1% Triton X-100 and collected into scintillation vials. For measuring the uptake in the absence of sodium, Tris–HCl buffer (pH 7.4) with 137 mM choline

Table 1. Effect of clonidine on cAMP production in bovine adrenal chromaffin cells

	cAMP (pmol/mg protein)	% of Control
Control	13.2 ± 0.8	100
Forskolin (1 μM)	28.6 ± 2.7*	216
Norepinephrine (100 μM)	13.9 ± 0.7	107
Clonidine (100 μM)	11.2 ± 0.7	85
Forskolin + clonidine	40.8 ± 9.5†	300

Cells were incubated for 10 min at 37° in the presence of drugs. Values are means ± SEM of two experiments, each done in triplicate.

* $P < 0.001$ compared to control.

† $P < 0.01$ compared to the clonidine group.

chloride was used for incubation. The amount of clonidine taken up by the cells was calculated from the initial concentration of clonidine and the results are expressed as nmol clonidine/10^6 cells.

Materials

[^{3}H]Clonidine (sp. act. 66 Ci/mmol) was obtained from New England Nuclear. The radioimmunoassay kits for cAMP and cGMP, ^{45}CaCl (sp. act. 21 mCi/mg) and [^{3}H]*myo*-inositol (sp. act. 82.5 Ci/mmol) were purchased from the Amersham Corp. The following drugs were purchased: clonidine from Research Biochemical Inc., oxymetazoline, naphazoline, and calimidazoline (R24571) from the Sigma Chemical Co.; and GTPγS and forskolin from Calbiochem. (±)-Idazoxan (Reckitt-Coleman, England) and SKF 86466 (Smith Kline & French, U.S.A) were gifts from these companies.

RESULTS

Effects of clonidine on production of cAMP and cGMP, on PI turnover, and on influx of calcium into adrenal chromaffin cells

Effect on production of cAMP. Forskolin (1 μM) elicited a significant stimulation, by over 2-fold, of the production of cAMP in adrenal chromaffin cells whereas norepinephrine was not effective (Table 1). Clonidine (100 μM) neither affected basal production nor inhibited the stimulation by forskolin of cAMP. On the other hand, in rat cerebral cortex slices, clonidine significantly inhibited the production of cAMP, stimulated by either forskolin or norepinephrine (basal, 4.1 ± 0.3 pmol/mg protein; forskolin [50 μM], 21.9 ± 4.9; norepinephrine, 14.4 ± 0.4; forskolin + clonidine, 10.3 ± 1.1, $P < 0.01$; norepinephrine + clonidine, 10.6 ± 0.21, $P < 0.01$). These results, therefore, indicate that the effects of clonidine in adrenal chromaffin cells differ from a typical response (inhibition) to stimulated α_2-adrenergic receptors, as shown here for cAMP using rat cerebral cortex as the model tissue.

Effect on PI turnover. Carbachol and GTPγS, an activator of phospholipase C, both substantially increased, whereas norepinephrine did not modify, the turnover of PI in chromaffin cells (Table 2). Clonidine had no effect on basal turnover of PI nor did it modify the effect of carbachol or GTPγS. This finding suggests that the clonidine binding site in adrenal chromaffin cells is not coupled to PI turnover.

Effect on production of cGMP. Clonidine (100 μM) produced a significant increase in cGMP levels. A significant response was detectable at 5 min, peaked at 15–20 min after stimulation, and then declined (Fig. 1A). This time course for the increase in cGMP elicited by clonidine was slower than that reported for the effect of acetylcholine which also increases cGMP production in adrenal chromaffin cells [20]. The response was also concentration-dependent, apparent at 0.1 μM and reaching a maximum at approximately 50 μM (Fig. 1B).

We also compared the effects of clonidine and acetylcholine on accumulation of cGMP in the presence and absence of 3-isobutyl-1-methylxanthine (IBMX), and inhibitor of phosphodiesterase, since the receptor-mediated elevations in cGMP are usually potentiated by inhibition of this enzyme [20]. As shown (Table 3), clonidine significantly increased cGMP by 1.5-fold while acetylcholine increased it by 3-fold. IBMX enhanced the amount of cGMP formed in response to clonidine (56 to 80%) and to acetylcholine (from 211 to 285%).

Table 2. Effect of clonidine on PI turnover in bovine adrenal chromaffin cells

	IP formed (% of total dpm)	% of Control
Control	3.3 ± 0.2	100
Carbachol (100 μM)	8.1 ± 0.2*	245
GTPγS (50 μM)	9.2 ± 0.4*	278
Norepinephrine (100 μM)	3.6 ± 0.2	109
Clonidine (100 μM)	3.2 ± 0.2	97
Clonidine + carbachol	8.0 ± 0.3†	242
Clonidine + GTPγS	9.3 ± 0.3†	281

The hydrolysis of PI was measured for 30 min in chromaffin cells as described. The amount of inositol-1-phosphate formed is expressed as percent of inositol-1-phosphate. In a typical control experiment, values for dmp/10^6 cells of inositol-1-phosphate and lipid fractions were 1,100 ± 150 and 25,000 respectively. Values are means ± SEM of two experiments performed in triplicate.

* $P < 0.001$ compared to control.

† $P < 0.001$ compared to the clonidine group.

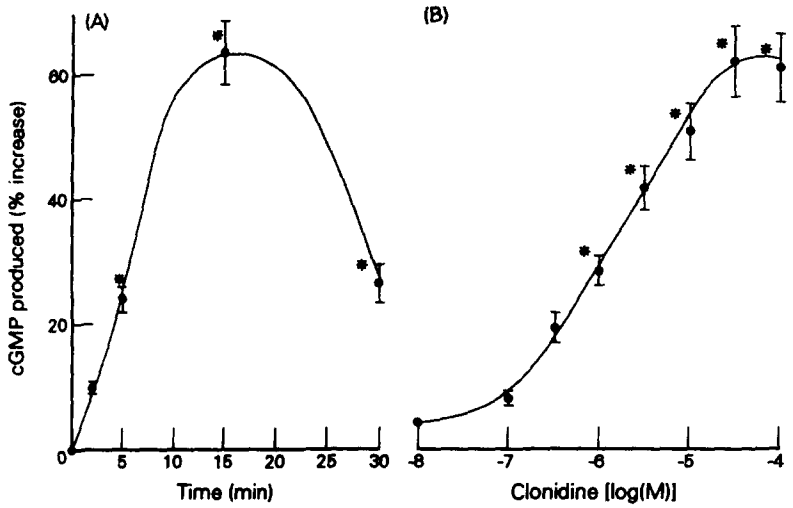

Fig. 1. Time and concentration-dependent effects of clonidine on cGMP production in chromaffin cells. The total amount of cGMP produced in the presence of IBMX was measured by radioimmunoassay. (A) Cells were incubated for various times in the presence of 100 μM clonidine. The amount of cGMP produced without clonidine was 25.6 ± 2.3 pmol/mg protein. Each point is the mean of two experiments carried out in triplicate. (B) Cells were incubated for 15 min with various concentrations of clonidine. Each point is the mean ± SEM of triplicates from three experiments. Key: (*)$P < 0.01$ compared to control.

Effect on influx of calcium. Clonidine (100 μM) and carbachol (100 μM) elicited a significant increase in the uptake of $^{45}Ca^{2+}$ into adrenal chromaffin cells (Table 4). The influx of $^{45}Ci^{2+}$ was significantly higher than control at 30 sec and reaching a maximum at 1 min; the difference between basal and clonidine-stimulated influx declined after 1 min (Fig. 2). The response was concentration dependent with maximal responses (about 170% over basal) obtained with 10 μM clonidine (Fig. 3). Moreover, as shown in Table 4, the increase in the influx of $^{45}Ca^{2+}$ was observed only when cells were prelabeled with $^{45}Ca^{2+}$ for at least 1 min before the addition of clonidine. On the contrary, carbachol increased the influx of $^{45}Ca^{2+}$ with or without prelabeling with $^{45}Ca^{2+}$.

Effects of other agents interacting with IRs on production of cGMP and influx of calcium into adrenal chromaffin cells

Effects on cGMP production. To ascertain the specificity of the increase in cGMP elicited in adrenal chromaffin cells by clonidine, we examined the effects of other agents which are known to bind to IR. These included oxymetazoline, naphazoline, idazoxan [21], and rilmenidine [2]. Since all of these agents also bind to α_2-adrenergic receptors [21], we also examined the effects of norepinephrine. In contrast to clonidine, none of these agents increased cGMP production (Table 5). Moreover, the effect of clonidine was not blocked by idazoxan (300 μM), an α_2-antagonist which also binds to IR. These findings suggest that the accumulation of cGMP elicited by clonidine is not shared by other agents that bind to IRs and, moreover, may not be mediated by typical cell surface receptors, either α_2-adrenergic or IRs.

Table 3. Effect of clonidine on cGMP production in bovine adrenal chromaffin cells

	cGMP (pmol/mg protein)	
	No IBMX	With IBMX
Control	13.3 ± 1.3	27.5 ± 5.9
Clonidine (100 μM)	20.8 ± 2.2*	49.6 ± 8.2*
Acetylcholine (100 μM)	41.3 ± 3.5†	106 ± 5.0†

Cells were incubated for 15 min at 37° in the presence or absence of IBMX (0.3 mM). Values are means ± SEM of two separate experiments, each done in quadruplicate.
* $P < 0.01$ compared to control.
† $P < 0.001$ compared to control.

On the other hand the specific action of clonidine may reflect an intracellular action as with some other imidazoles, such as RO 20-1724, which act directly on a cGMP phosphodiesterase [22]. To ascertain whether clonidine was accumulated intracellularly, we measured the uptake of [^{3}H]clonidine into chromaffin cells.

Adrenal chromaffin cells took up [^{3}H]clonidine in a concentration-dependent manner, with approximately 300 pmol/10^6 cells in 15 min using 1 mM [^{3}H]clonidine (data not shown). The uptake was nonsaturable and not modified by sodium-free medium. Moreover, ouabain, norepinephrine and cocaine (all 100 μM) failed to inhibit clonidine uptake. All these results indicate that the uptake of clonidine was due to a passive, nonspecific diffusion of clonidine into chromaffin cells, suggesting that certain effects of clonidine may be due to intracellular action.

Effects upon calcium uptake. We compared the

Table 4. Effect of clonidine on calcium influx into bovine adrenal chromaffin cells

	Calcium uptake (nmol/10^6 cells)	
	Prelabeled	Not prelabeled
Control	0.856 ± 0.05	0.318 ± 0.06
Clonidine (100 μM)	1.51 ± 0.12*	0.438 ± 0.07
Carbachol (100 μM)	2.65 ± 0.21*	0.945 ± 0.05*

The influx of $^{45}Ca^{2+}$ into cells was measured for 1 min following the addition of the agents shown. In "prelabeled" experiments, cells were preincubated for 1 min with $^{45}Ca^{2+}$ prior to the addition of clonidine or carbachol; in "not prelabeled" experiments, $^{45}Ca^{2+}$ was added along with the activating agent. Values are means ± SEM of two experiments, each done in triplicate.

* $P < 0.001$ compared to control.

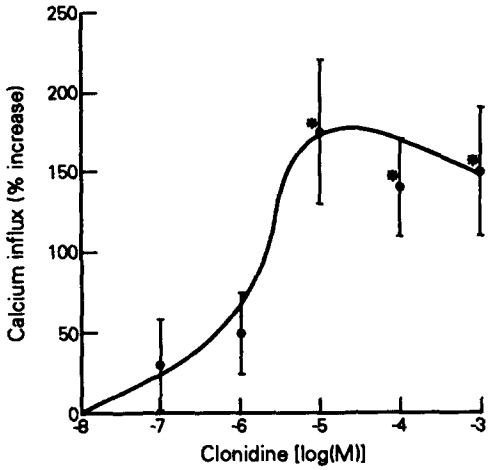

Fig. 3. Influx of $^{45}Ca^{2+}$ measured at different concentrations of clonidine after 1 min, preceded by a 1-min preincubation with $^{45}Ca^{2+}$. Control influx of $^{45}Ca^{2+}$ was 0.931 ± 0.09 nmol/10^6 cells. Values are means ± SEM from three experiments performed in triplicate. Key: (*)$P < 0.01$ compared to basal influx.

effects of clonidine on calcium uptake into adrenal chromaffin cells with several other agents which bind with IRs. In addition to clonidine, oxymetazoline, rilmenidine and naphazoline significantly increased the influx of calcium into adrenal chromaffin cells, with somewhat varying magnitudes (Table 6). Idazoxan which binds to IRs, however, did not increase calcium influx nor did it block the effect of clonidine. SKF-86466, a non-imidazole selective α_2-adrenergic antagonist [23], failed to modify the basal calcium uptake or to inhibit the increase produced by clonidine, but rather increased the effect of clonidine. Calimidazoline, an imidazoline derivative with potent Ca^{2+}-ATPase inhibitor activity [24], also increased the uptake of calcium.

DISCUSSION

Many substituted imidazoles and imidazolines, with or without adrenergic activity, exhibit biological activities in brain and peripheral tissues that are not related to their binding to adrenergic receptors. These include the central control of blood pressure [5], modulation of the release of catecholamines and expression of phenylethanolamine *N*-methyltransferase (PNMT) in the adrenal medulla [12, 25], the release of insulin by pancreatic islet cells [26], and the response of chemosensors in the carotid body [27]. Non-adrenergic binding sites that recognize the imidazole/imidazoline structure have been identified in brain, kidney, adrenal medulla, platelets and

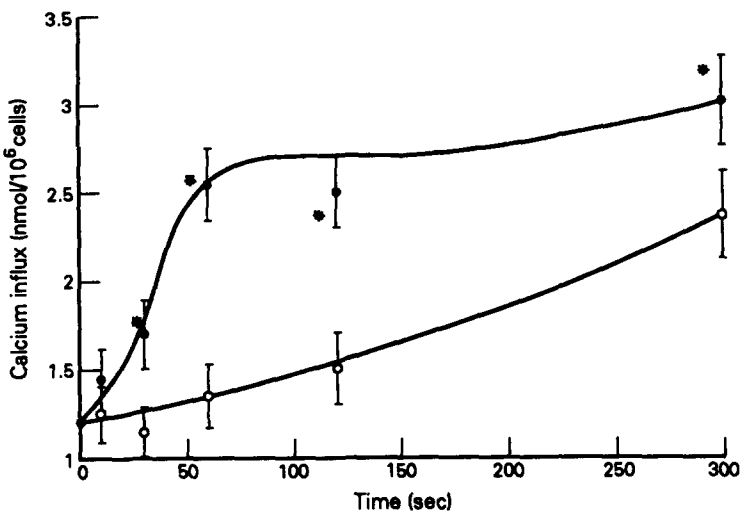

Fig. 2. Influx of $^{45}Ca^{2+}$ measured at different time points in the presence (●) or absence (○) of 100 μM clonidine. Cells were prelabeled with $^{45}Ca^{2+}$ for 1 min and clonidine was added to start the incubation. Values are means ± SEM from two experiments done in triplicate. (*)$P < 0.01$ compared to control uptake.

Table 5. Effects of clonidine and other imidazole receptor active agents on cGMP production in adrenal chromaffin cells

	cGMP (pmol/mg protein)	% of Control
Control	21.8 ± 1.12	100
Clonidine	34.1 ± 2.5*	156
Oxymetazoline	23.1 ± 2.8	106
Naphazoline	23.1 ± 2.5	106
Rilmenidine	25.2 ± 3.0	116
Idazoxan	26.4 ± 5.0	121
Clonidine + idazoxan	34.4 ± 4.7*	158
Norepinephrine	26.5 ± 2.6	122

Cells were incubated in KRB solution containing IBMX (300 μM) for 15 min. Concentrations of drugs were 100 μM. Values are means ± SEM of two experiments, each done in quadruplicate.

* $P < 0.01$ compared to control.

Table 6. Effects of clonidine and other imidazole receptor active agents on ^{45}Calcium influx into chromaffin cells

	^{45}Calcium (nmol/10^6 cells)
Control	0.573 ± 0.04
Clonidine	1.17 ± 0.21*
Oxymetazoline	0.989 ± 0.05*
Rilmenidine	1.45 ± 0.41*
Naphazoline	1.14 ± 0.10*
Idazoxan	0.659 ± 0.09
Clonidine + idazoxan	1.11 ± 0.05†
SKF-86466	0.624 ± 0.03
Clonidine + SKF-86466	1.92 ± 0.17*
Calimidazoline	1.73 ± 0.15*

The influx was measured for 1 min using 1 μCi $^{45}Ca^{2+}$. Concentrations of drugs used were 100 μM. Values are means ± SEM from at least two experiments, each done in triplicate.

* $P < 0.01$ compared to control.

† $P < 0.01$ compared to the clonidine group.

adipocytes [4, 9, 28, 29] and have led to postulation of a novel class of non-adrenergic receptors, IR. Further pharmacological studies have indicated the existence of at least two subclasses of IR, preferring either idazoxan or clonidine as ligands [21, 30]. Based on partial purification of the IR and studies on the expression of α_2-adrenergic receptors in neural cell lines, it has been fairly well established that the IR is physically distinct and differentially regulated from α_2-adrenergic receptors [8, 9].

In the present study, we sought to establish the signal transduction mechanism associated with occupancy of IRs. Such information strengthens evidence that IRs and α_2-adrenergic receptors are distinct. Moreover, it is of importance in understanding some of the biological functions of IRs and their regulation. To achieve these objectives, we examined the effects of clonidine and other agents which bind to IRs on the production of cAMP and cGMP, PI turnover, and accumulation of Ca^{2+} in primary cultures of bovine adrenal chromaffin cells. Chromaffin cells were selected because clonidine, a phenylimidazoline and prototype of a class of drugs interacting with IRs, has biological activity in these cells: it inhibits the release of catecholamines stimulated by cholinergic agonists and increases the content of mRNA for PNMT, effects which are not reversed by α_2-antagonists [12, 25]. Moreover, adrenal chromaffin cells express IRs on their cell surface [11].

We discovered that clonidine did not modify the basal levels of cAMP and failed to inhibit, in adrenal chromaffin cells, the stimulated production of cAMP by forskolin, the response usually produced by activation of α_2-adrenergic receptors [11]. However, in agreement with earlier observations in brain, clonidine significantly inhibited the forskolin- or norepinephrine-stimulated production of cAMP in rat cortical slices mediated by α_2-adrenergic receptors.

The absence of changes in cAMP in adrenal chromaffin cells in response to clonidine are consistent with the observations of Powis and Baker [12] and Feinland *et al.* [11] who failed to detect α_2-adrenergic receptors in the membranes of these cells, and helps to explain why clonidine's ability to inhibit release of catecholamines from chromaffin cells by cholinergic agonists is not reversed by α_2-antagonists [12]. It is also in agreement with our previous findings in whole rat adrenal gland [13]. While Wada *et al.* [31] have reported some high affinity specific α_2-adrenergic binding of [^{3}H]clonidine in chromaffin cell membranes, the inability of clonidine to alter cAMP production suggests that, even if present, these "receptors" are not functionally coupled to the adenylate cyclase system.

Clonidine failed to modify basal, carbachol- or GTPγS-stimulated hydrolysis of phosphatidylinositol by phospholipase C, a second messenger system that is linked to various neurotransmitter receptors [32]. The absence of any effect of clonidine on basal or stimulated PI turnover indicates that IR sites in chromaffin cells are not coupled either positively or negatively to the PI system, which is in agreement with the finding that in rat cerebral cortex clonidine fails to inhibit the turnover of PI stimulated by carbachol [33]. It also demonstrates that the action of clonidine to inhibit the carbachol-stimulated synthesis and release of catecholamines [4, 33] is not linked to inhibition of PI hydrolysis.

On the other hand, clonidine (100 μM) produced a significant increase in cGMP in adrenal chromaffin cells. This observation was of interest in view of the facts that cGMP itself modulates catecholamine release [34] and that muscarine and atrial natriuretic factor, agents that inhibit catecholamine release from chromaffin and PC12 cells, increase cGMP levels in those tissues [20, 34].

Nonetheless, the change in cGMP accumulation elicited in chromaffin cells by clonidine differed in several respects from the well-known elevation of this cyclic nucleotide by acetylcholine [20]. In comparison, the response of cGMP to clonidine was: (a) smaller in magnitude—only 25% of that elicited by acetylcholine; and (b) slower in onset. Moreover,

the elevation of cGMP by clonidine was not replicated by other agents which bind with high affinity to IRs (as well as to α_2-adrenergic receptors) including oxymetazoline, rilmenidine and naphazoline; and was not blocked by idazoxan, a potential antagonist of IRs as well as α_2-adrenergic receptors. Hence, the action of clonidine in elevating cGMP appears not to represent an event at cell-surface IRs, but rather may be a particular property of clonidine itself.

In fact, the characteristics of the elevation of cGMP produced by clonidine in chromaffin cells are consistent with those of agents, such as sulmazole, which elevate cGMP by inhibiting specific cGMP phosphodiesterase intracellularly [35]. Supporting this view, i.e. that clonidine may be acting intracellularly, are the results of our uptake experiments which demonstrate that chromaffin cell membranes are highly permeable to clonidine, permitting the drug to be accumulated within the cell presumably by passive diffusion. Also, it has been demonstrated that some imidazoles inhibit cyclic nucleotide phosphodiesterases by direct action [22].

While the data indicate, therefore, that the receptors do not appear to be coupled to cAMP, cGMP or PI turnover, we observed that clonidine significantly increased the basal uptake of calcium into adrenal chromaffin cells. The changes in calcium flux elicited by clonidine, somewhat smaller than those elicited by carbachol, were detected under conditions in which the cells were preincubated with $^{45}Ca^{2+}$ prior to activation of uptake by addition of clonidine. Unlike the effects of clonidine upon cGMP accumulation, the effects on calcium uptake were shared by other agents known to bind to IRs including oxymetazoline, rilmenidine, and naphazoline. The fact that the increase in calcium uptake elicited by clonidine was not blocked by the selective α_2-adrenergic antagonist SKF-86466 provides further evidence that adrenergic mechanisms were not responsible. The clonidine-mediated increase in calcium uptake also was not inhibited by idazoxan, an α_2-antagonist that binds to IR.

These findings suggest that binding to IRs on chromaffin cells increases the uptake of calcium, although clear agonist–antagonist relationships—such as with idazoxan—to the IR remain to be established. The fact that equilibration of the cells with $^{45}Ca^{2+}$ was required to observe this response may be an indication that there is a primary effect which must occur, then resulting in a secondary redistribution of calcium. The data also suggest that the inhibition of catecholamine release and calcium influx by clonidine observed under stimulating conditions [12] and the moderate increase in calcium entry observed in the present study under basal conditions represent two independent mechanisms.

The present study, therefore, clearly demonstrates that the signal transduction mechanisms coupled to IRs in adrenal chromaffin cells are functionally distinct from classical G-protein coupled second messenger systems and, in particular, those coupled to α_2-adrenergic receptors. These observations strongly support the abundant data from ligand binding [21, 23, 28], biochemical [8], and the present functional studies, demonstrating that the two classes of receptors are distinct.

Our findings, moreover, suggest that the IR may be coupled to or part of a ligand-gated ion channel system: imidazole agents primarily or secondarily gate the influx of calcium into the cell. Consistent with this view are studies by others that report effects of imidazole-like agents on ion regulating systems. Some of these, such as cirazoline and idazoxan, and also an endogenous clonidine displacing substance, modulate $^{22}Na^+$ influx in kidney proximal tubule cells, independent of the stimulation of α_2-adrenergic receptors [8, 36]. In addition, clonidine has been shown to inhibit the Na^+-H^+ exchange system in placental membranes [37], as well as in an epithelial cell line [38]. Furthermore, the inhibition of IR binding by K^+-channel blockade has been reported recently to occur in rat hepatocytes [39], suggesting that K^+-gating may be associated with IR activation. Calimidazoline, a potent inhibitor of Ca^{2+}-ATPase, as we have demonstrated here, increases calcium influx and appears to bind to IR (unpublished observation). Future investigations must target the ionic mechanisms linked to IRs, in particular Na^+-H^+ or Na^+-Ca^{2+} exchange systems, K^+ channels, and Ca^{2+} channels—not only in adrenal chromaffin cells, but in other tissues that express IRs.

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