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ACCELERATED COMMUNICATION

Clonidine-Displacing Substance from Bovine Brain Binds to Imidazoline Receptors and Releases Catecholamines in Adrenal Chromaffin Cells

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SUMMARY

Identification of nonadrenergic binding sites for clonidine and related imidazolines in brain and peripheral tissues and partial purification of an endogenous ligand for these sites have led to the postulation of a novel transmitter/receptor system. The receptors seem to be present in adrenal medulla and to regulate chromaffin cell function. The present study was undertaken to test the ability of the putative endogenous ligand clonidine-displacing substance (CDS) to displace [^{3}H]idazoxan binding to adrenal chromaffin cell membranes and to release catecholamines from cultured chromaffin cells. CDS potently displaces [^{3}H]idazoxan binding to chromaffin cell membranes, with an IC_{50} of 5 units. The displacement of [^{3}H]idazoxan binding by CDS was not modified by guanosine 5′-(β,γ-imido)triphosphate, suggesting that the imidazoline binding sites may not be GTP-binding protein-coupled receptors. CDS produced a large release of catecholamines from chromaffin cells, and the release was partially blocked by cobalt, a calcium channel blocker. The calcium-dependent release reached a plateau above 5 units of CDS, with a maximal response at 15 min. It is concluded that endogenous CDS, prepared from brain, regulates the secretion of catecholamines from adrenal chromaffin cells, probably by activating imidazole receptors.

It is now recognized that clonidine and other structurally related imidazoles and imidazolines bind not only to α_2-adrenergic receptors but also to a novel class of receptors variously termed imidazole-preferring receptors (1), imidazoline-guanidinium-receptive sites (2), or simply, as we shall refer to them, IRs. IRs differ from α_2-adrenergic receptors structurally, functionally, and with respect to distribution among and within organs, including regionally in brain (2–5). Present evidence suggests that, like many other receptor types, IRs are composed of more than one subclass, based on rank order of binding of different ligands, with one subclass that preferentially binds idazoxan and one that preferentially binds clonidine or its congener *p*-aminoclonidine (6). The mode of the intracellular function of IRs is unclear, although it appears to involve movement of calcium in the case of chromaffin cells (4). Of great interest is the recent demonstration that IRs may be localized intracellularly in platelets and kidney (3, 7) and on mitochondrial membranes in liver (8).

The native ligand for the IR is unknown. Binding studies have demonstrated that it is not histamine, the principal biologically active imidazole-containing substance in vertebrates. Recently, a substance of low molecular weight, termed CDS, has been isolated and partially purified from bovine brain (9, 10). Because it displaces clonidine from brain membranes, it was originally proposed to be a native inhibitor of catecholamine at α_2-adrenergic receptors (9). However, the demonstration that it binds with high affinity to IR (11) has raised the possibility that it may be an endogenous ligand for these receptors.

Although CDS has been demonstrated to modify arterial pressure when injected into brain (11), to inhibit platelet aggregation (12), and to inhibit the contraction of the electrically stimulated vas deferens (13), these activities may also involve interactions with α_2-adrenergic receptors. Moreover, it is not certain from these studies whether CDS acts as an agonist or antagonist at IRs. The possibility that it may be an agonist has been suggested by the demonstration that CDS stimulates contraction of the rat gastric fundus (14), yet it is not certain whether the effect in that tissue is directly on cells containing the receptor.

In our initial studies, we have observed that chromaffin cells of bovine adrenal medulla express IRs but not α_2-adrenergic

ABBREVIATIONS: IR, imidazole/imidazoline receptor; CDS, clonidine-displacing substance; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; NE, norepinephrine; G protein, GTP-binding protein; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate.

receptors, making them useful for studying the intracellular effects of IR occupancy. In the studies described here, we have sought to determine whether CDS binds to IRs of chromaffin cells and whether the interaction stimulates the release of catecholamines. If so, the finding would provide persuasive evidence that CDS has a biological action at the cellular level.

Materials and Methods

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*. Glands were perfused with collagenase (Worthington) and DNase (Sigma Chemical Co.), and the medullae were dissociated from cortex. The tissue was minced, digested in collagenase for 40–60 min, and filtered through a 105-μm wire sieve. The 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 gradients, washed, and plated at required density in Dulbecco's modified Eagle's medium/F12 (1:1) supplemented with 10 mM HEPES (pH 7.4), 10% fetal calf serum, and 0.1% antibiotics (penicillin and streptomycin). The cells were studied between day 3 and 5 of culture.

[^{3}H]Idazoxan binding to chromaffin cell membranes. Washed cells (ice-cold 50 mM HEPES with 265 mM sucrose, pH 7.4) were suspended in 50 mM ice-cold Tris·HCl buffer (pH 7.4), with 5 mM EDTA, and homogenized using a Polytron (Brinkman) (setting 6), for 10 sec. The homogenate was centrifuged at 40,000 × *g* for 30 min at 4°. The resulting pellet was washed three times by resuspension in Tris·HCl buffer and recentrifugation. The final washed pellet was suspended in fresh Tris·HCl buffer (pH 7.4), to give approximately 200–300 μg of protein/assay tube, with a final volume of 1 ml, and was incubated at 25° for 45 min with [^{3}H]-idazoxan. Saturation binding was performed using 0.1–15 nM [^{3}H]-idazoxan. Because cirazoline exhibited higher affinity for IR than did idazoxan (27), the nonspecific binding was defined using 10 μM cirazoline. Incubations were terminated by vacuum filtration over Whatman GF/B filters, using a modified cell harvester (Brandel). The filters were washed with 10 ml of ice-cold buffer and suspended in scintillation cocktail, and the radioactivity was determined by liquid scintillation counting. Binding data were analyzed in greater detail using LIGAND (Elsevier Biosoft), a parametric nonlinear regression routine.

Release of catecholamines. Cells (1 × 10^6/well; six-well plates) were washed twice with Krebs-Ringer bicarbonate buffer (pH 7.4). The release of catecholamines was initiated by incubating the cells at 37°, in a total volume of 1 ml of Krebs-Ringer-bicarbonate buffer, with stimulating agent. After the specified time, the medium was rapidly removed and immediately stored at −70° for assay of catecholamines by HPLC. The cells were harvested using 0.4 M perchloric acid, and the precipitated protein was removed by centrifugation.

Catecholamine content of the acid extract was determined by precolumn treatment with alumina, followed by HPLC analysis (17). Briefly, the catecholamines were separated using a reverse phase C_{18} column (Waters) and isocratic elution with 0.15 M monochloroacetate buffer (pH 3.0) containing 2 mM EDTA and 1 mM sodium octylsulfate. The catecholamines were detected using an LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN), at a potential of +0.65 V, versus a Ag/AgCl reference electrode. Samples were filtered, using Millex filters (0.45-μm; Millipore), before injection into the system. The results are expressed as the percentage released into the medium, relative to the total cell content. The cell content of epinephrine and NE was 22.6 ± 2.4 nmol/10^6 cells and 16.9 ± 2.1 nmol/10^6 cells, respectively.

Isolation of CDS. CDS was prepared by methods developed earlier in this laboratory (10), with subsequent modifications to isolate CDS with increased purification from synaptosomes rather than whole brain (18). Briefly, a crude P_2 pellet was prepared from bovine brain and osmotically shocked by resuspension of in 7.5 volumes (v/v) of distilled water (Polytron, setting 4, 10 sec). The suspension was centrifuged at 37,000 × *g* for 15 min. The supernatant was then heat denatured and centrifuged at 100,000 × *g* for 30 min. The resulting supernatant was lyophilized and dialyzed (1000 molecular weight cut-off); the diffusate was dried and then extracted with methanol, as described previously (10). Samples of redried methanol extract containing CDS were stored at −70° and reconstituted in buffer just before use.

For a limited number of experiments, CDS was further purified by ion exchange chromatography on a Mono Q HPLC column (Pharmacia), with a linear gradient of 0.02–0.10 M potassium phosphate. Fractions with CDS-like activity were determined by radioreceptor assay, pooled, concentrated, and loaded onto an immunoaffinity column prepared from anti-*p*-aminoclonidine IgG (19). CDS was specifically eluted at high pH with diethylamine. Samples prepared in this manner are termed "affinity-purified" CDS and were concentrated and stored at −70° until use. The purification scheme for CDS is described elsewhere in greater detail.[1]

One unit of CDS is defined as the amount of CDS that produces 50% inhibition of *p*-[^{3}H]aminoclonidine binding to brain frontal cortex membranes (10).

Materials. [^{3}H]Idazoxan (43 Ci/mmol) and *p*-[^{3}H]aminoclonidine (50 Ci/mmol) were purchased from Amersham Corporation and New England Nuclear, respectively. Cirazoline and SKF-86466 (6-chloro-*N*-methyl-2,3,4,5-tetrahydrol-1*H*-3-benzapine) were gifts from Synthelabo (Paris) and Smith Kline & French (King of Prussia, PA), respectively. Rauwolscine was purchased from Research Biochemical, Inc. (Boston, MA).

Results

Interaction of CDS with [^{3}H]idazoxan binding to adrenal chromaffin cell membranes. The binding of [^{3}H] idazoxan to membranes of adrenal chromaffin cells (Fig. 1) was specific, saturable, and of high affinity (K_D, 4.6 nM). The binding was not inhibited by the selective α_2-adrenergic agent rauwolscine but was totally displaced by unlabeled idazoxan, an imidazoline (Fig. 2A). These findings are in accord with conclusions of other studies that adrenal chromaffin cells may not express functionally active α_2-adrenergic receptors (4) and,

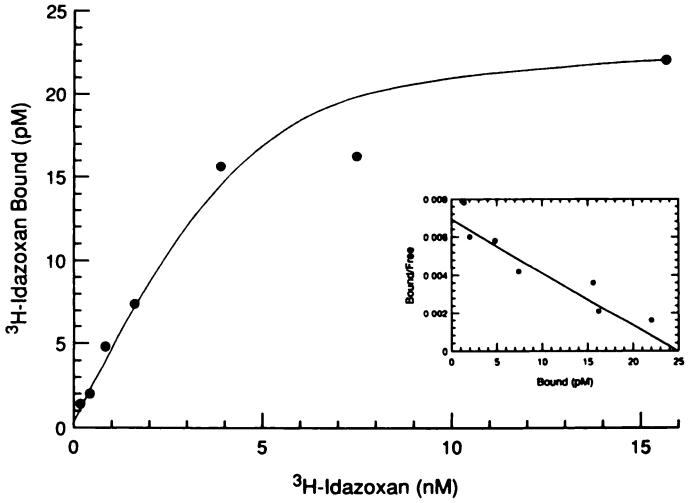

Fig. 1. Saturation isotherm of specific [^{3}H]idazoxan binding to chromaffin cell membranes. The nonspecific binding was defined using 10 μM cirazoline. *Inset*, Scatchard plot of specific binding.

[1] M. P. Meeley *et al.* Manuscript in preparation.

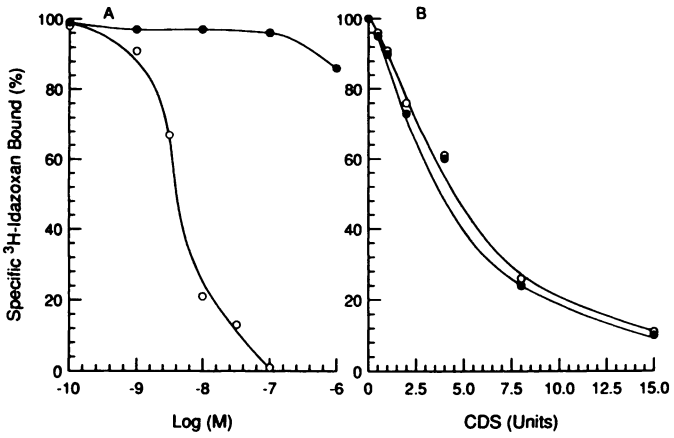

Fig. 2. A, Inhibition of [^{3}H]idazoxan binding to adrenal chromaffin cell membranes by idazoxan (○) and rauwolscine (●). B, Inhibition of [^{3}H] idazoxan binding by CDS in the absence (○) and presence (●) of 50 μM Gpp(NH)p. Membranes were incubated at 25° for 45 min with 5 nM [^{3}H] idazoxan; nonspecific binding was defined using 10 μM cirazoline. The total counts were about 2500 dpm; the nonspecific binding averaged 30% of total binding.

TABLE 1

Effect of CDS on catecholamine release from bovine adrenal chromaffin cells

The amount of catecholamines released from chromaffin cells (1 × 10^6 cells) in 10 min in the presence of CDS was measured. The catecholamine content of cells was 22.6 ± 2.4 and 16.9 ± 2.1 nmol/10^6 cells for epinephrine and NE, respectively. Values are mean ± standard error of two experiments done in triplicate.

	Epinephrine	NE
	% of cell content	*% of cell content*
Control	0.84 ± 0.11	1.02 ± 0.13
Nicotine (10 μM)	18.2 ± 1.5[a]	28.0 ± 2.5[a]
CDS (10 units)	18.1 ± 1.4[a]	7.6 ± 1.2[a]
Affinity-purified CDS (10 units)	18.3 ± 1.6[a]	9.6 ± 0.93[a]
SKF-86466 (100 μM)	0.95 ± 0.13	0.99 ± 0.11
Nicotine + hexamethonium (100 μM)	1.6 ± 0.1[b]	2.4 ± 0.3[b]
Nicotine + cobalt (1 mM)	0.95 ± 0.11[b]	0.71 ± 0.10[b]
CDS (10 units) + hexamethonium (100 μM)	17.1 ± 1.5[a]	7.5 ± 1.3[a]
CDS (10 units) + cobalt (1 mM)	15.9 ± 0.8[c]	3.6 ± 0.4[c]
CDS (10 units) + SKF-86466	17.5 ± 1.1[a]	8.1 ± 0.9[a]

[a] $p < 0.001$, compared with basal release.
[b] $p < 0.001$, compared with nicotine group.
[c] $p < 0.001$, compared with CDS group.

hence, that the observed binding of [^{3}H]idazoxan to adrenal membranes is exclusively to IRs.

CDS, isolated from bovine brain synaptosomes, completely displaced [^{3}H]idazoxan binding from adrenal chromaffin cell membranes, with an IC_{50} of approximately 5 units (see unit defined in Materials and Methods) (Fig. 2B). The binding of CDS to adrenal membranes was not modified by Gpp(NH)p (Fig. 2B), a nonhydrolyzable analog of GTP that has been shown to decrease the affinity of agonist binding to G protein-coupled receptors (20). This initial observation supports the view that IRs in adrenal chromaffin cells, similarly to those in kidney (21) and liver (22), may not be coupled to G proteins.

Effect of CDS on catecholamine release. Incubation of CDS with adrenal chromaffin cells elicited a substantial release of epinephrine and NE (Table 1; Figs. 3 and 4). Analysis of the time-course of release (Fig. 3) demonstrated that the onset was almost instantaneous and that 50% of the release occured within the first 2 min, followed by a gradual increase over the

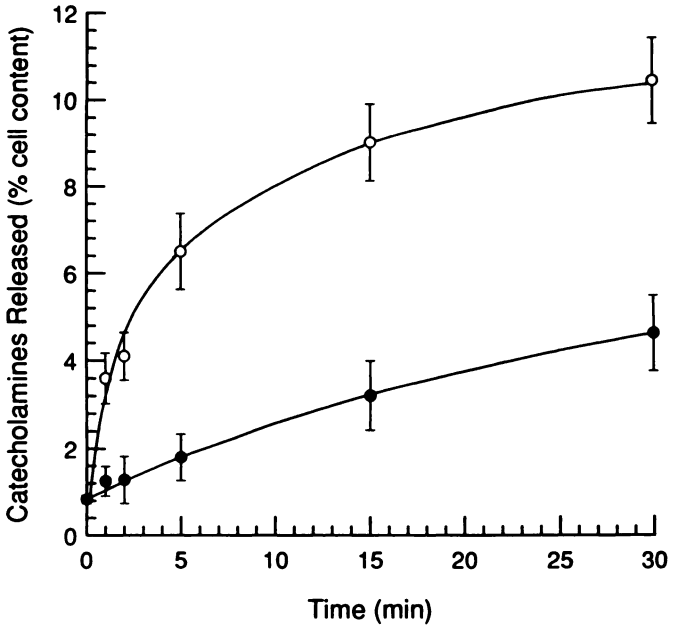

Fig. 3. Time-course of catecholamine release from chromaffin cells in the presence (○) and absence (●) of 5 units of CDS. Each value is the mean ± standard error of two experiments done in triplicate. Release at all time points was significantly different ($p < 0.001$) from the corresponding basal release.

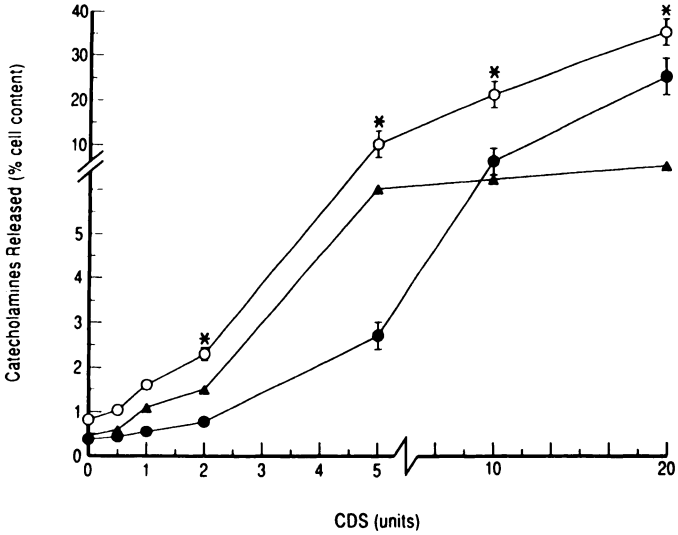

Fig. 4. CDS-stimulated catecholamine release from adrenal chromaffin cells after 10 min in the presence (●) and absence (○) of 1 mM cobalt; ▲, Difference between the presence and absence of cobalt. Each value is the mean ± standard error of two experiments done in triplicate. *, $p < 0.001$, compared with corresponding cobalt group.

next 25 min. The time-course of the CDS-evoked release of catecholamines was similar to that elicited from chromaffin cells by other secretagogues (23), suggesting that the secretory mechanism engaged by CDS may share common properties with those of other agents.

CDS was more effective in releasing epinephrine than NE (Table 1). Thus, at rest, the release of NE and epinephrine was approximately equal, with a NE/epinephrine ratio of 1.2:1. However, 10 units of CDS had a more potent effect upon epinephrine than NE, increasing the release of epinephrine 21-fold, whereas NE release was increased only 7.5 times. The

NE/epinephrine release ratio was, therefore, reduced in the presence of CDS to 0.42 (Table 1).

The release of catecholamines elicited by CDS was concentration dependent (Fig. 4). A significant elevation was obtained at 2 units, and release continued to increase progressively up to the maximum tested (20 units). The effect of CDS prepared from synaptosomes was reproduced using affinity-purified CDS (Table 1). This indicates that the effect of CDS on release was probably not due to secretagogues co-extracted in crude synaptosomal preparations.

Nicotine elicited a well characterized (23) dose-dependent release of catecholamines from adrenal chromaffin cells (Table 1). The response was near-maximal at 10 μM, with a time-course comparable to that evoked by CDS (data not shown). Nicotine-stimulated release was almost completely blocked by the nicotinic cholinergic antagonist hexamethonium (Table 1). In contrast to CDS, nicotine preferentially released more NE than epinephrine; the increase in NE was 38-fold, whereas that of epinephrine was 21-fold, an elevation comparable to that elicited by 10 units of CDS (Table 1). The secretion of catecholamines elicited by CDS was not antagonized by hexamethonium (Table 1), indicating that the effect of CDS is independent of nicotinic cholinergic receptor activation. In addition, the selective α_2-antagonist SKF-86466 (24), which does not bind to IRs (18), failed to inhibit the CDS-stimulated release of catecholamines (Table 1).

Relationship to calcium. We investigated whether extracellular calcium was required for the CDS-elicited release of catecholamines from chromaffin cells, by comparing the effect of cobalt on the catecholamine-secretory actions of CDS and nicotine, a secretagogue whose actions depend upon calcium entry (24) (Table 1). The release of catecholamines by nicotine was completely blocked by addition of 1 mM Co^{2+}, an ion that blocks the stimulated entry of calcium (25). However, 1 mM Co^{2+} only partially reduced the release of catecholamines by CDS. When examined over a range of concentrations of CDS (Fig. 3), 1 mM Co^{2+} shifted the dose/release response curve to the right. Thus, cobalt totally blocked the release of catecholamines effected by up to 5 units of CDS, whereas at higher doses it partially reduced, but did not abolish, release. The calcium-dependent release (the difference in release in the presence and absence of cobalt) reached a plateau at and above 5 units of CDS.

Discussion

In initial experiments, we observed that [^{3}H]idazoxan binds to membranes of chromaffin cells with high affinity and that this ligand cannot be displaced by the selective α_2-adrenergic antagonist rauwolscine (26). Moreover, idazoxan binds to chromaffin cell membranes with a higher affinity than clonidine, *p*-aminoclonidine, and several other imidazoles (27), indicating that the IRs of the adrenal medulla may be of the so-called idazoxan-preferring subclass (6). In general, these observations add further support for the conclusions of both physiological experiments (28) and analysis of second messenger mechanisms (4), that adrenal chromaffin cells do not express the α_2-adrenergic receptor.

CDS, like unlabeled idazoxan, fully displaces [^{3}H]idazoxan from adrenal chromaffin cell membranes, with an IC_{50} of about 5 units. Thus, CDS appears to interact with IRs of the idazoxan subclass (6) in adrenal membranes. CDS has also been shown to bind to *p*-[^{3}H]aminoclonidine-preferring sites in brain and kidney (11, 18) and to [^{3}H]idazoxan sites in kidney and liver (21, 22). Thus, it is probable that CDS, an endogenous ligand, interacts with IRs of both purported idazoxan and clonidine subclasses (6).

In the present study, we have discovered that CDS, obtained from synaptosomes of bovine brain, is a potent secretagogue for adrenal chromaffin cells, releasing both epinephrine and NE. It is highly unlikely that the effect can be attributed to some other secretagogue co-purifying with CDS, because CDS obtained by separation on ion exchange HPLC, followed by immunoaffinity purification, elicited release of amounts of catecholamines from chromaffin cells comparable to those released by a similar dose of a synaptosomal extract containing CDS. Because, presumably, such purification eliminates contamination by other secretagogues, the catecholamine-releasing actions of both synaptosomal and affinity-purified material can be attributed to CDS.

The release of catecholamines from adrenal chromaffin cells by CDS was dose dependent and exhibited a time-course similar to that of other known adrenomedullary secretagogues (23). Interestingly, a maximum response was never reached with increasing amounts of CDS, at least within the concentration range tested. Indeed, CDS appears to differ in this respect from most other agents, including K^+ and nicotine. The release of catecholamines by CDS also differed from that produced by nicotine with respect to the profile of catecholamine release. Chromaffin cells are differentiated with respect to their capacity to synthesize and store catecholamines, in part related to their capacity to express the epinephrine-synthesizing enzyme phenylethanolamine-*N*-methyl transferase. The possibility that the two classes of chromaffin cells (NE- or epinephrine-containing) may be selectively stimulated by differential neuronal or chemical signals has often been proposed (29). The possibility, therefore, exists that epinephrine-containing chromaffin cells may express and/or be more potently regulated through IRs than those that contain NE.

Consistent with our binding studies, the ability of CDS to release catecholamines is not inhibited by a selective α_2-antagonist, SKF-86466, clearly establishing the absence of any α_2 receptor activation by CDS. We have recently demonstrated that drugs that bind to the IR in adrenal chromaffin cells do not stimulate or inhibit the accumulation of cAMP, the turnover of inositol trisphosphate, or, for the most part, the accumulation of cGMP (4). These findings have suggested that in adrenal medulla, as is the case in kidney and liver (21, 22), the IR may not be a G protein-coupled receptor. This conclusion is supported here by the demonstration that the kinetics of binding of CDS to the IR in chromaffin cell membranes are not modified by Gpp(NH)p.

On the other hand, stimulation of the IR on adrenal chromaffin cells does increase the transport of calcium from the medium, possibly secondarily to activation of some other ligand-gated ion channel or transporter (4). The fact that the intracellular transport of calcium stimulated by the IR is biologically relevant has been demonstrated here by the finding that the secretion of catecholamines stimulated by CDS, as with nicotine and other adrenomedullary secretagogues, depends, at least in part, upon the presence of extracellular calcium. That is, addition of 1 mM Co^{2+} to the incubation

medium markedly attenuated or abolished the secretory response to CDS as well as to nicotine.

In summary, this study provides the most direct evidence to date, at a cellular level, that CDS has a biological action. It demonstrates that CDS binds to the IR on adrenal chromaffin cell membranes. As a consequence, CDS also influences the entry of calcium, directly or indirectly, and stimulates release of catecholamines. The physiological role of this mechanism, however, remains a mystery. Some other imidazoline agents also stimulated the release of catecholamines from chromaffin cells (27), and further work is in progress to classify these agents as agonists or antagonists at the IR, based on this functional activity.

The source of CDS detected in serum (30) appears to be the adrenal gland, because CDS in the circulation was abolished in adrenalectomized rats.[2] The added fact that CDS binds to α_2-adrenergic receptors as well as IRs (11, 12) and may act centrally to regulate arterial pressure (10) suggests that it may play an important role in integrating widespread neural and endocrine networks involved in the regulation of blood pressure in health and disease.

Acknowledgments

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