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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

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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,

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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