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Tyrosine Hydroxylase Phosphorylation in Bovine Adrenal Chromaffin Cells

CLONIDINE STIMULATES BASAL BUT INHIBITS NICOTINIC RECEPTOR EVOKED PHOSPHORYLATION

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ABSTRACT. Clonidine inhibited the uptake of calcium and the overall phosphorylation of tyrosine hydroxylase induced by nicotinic receptor activation in bovine adrenal medullary chromaffin cells in culture. However, clonidine did not inhibit the increase in these parameters that accompanied K^+ depolarisation of the cells. There was also no effect of clonidine on the overall phosphorylation of tyrosine hydroxylase when cells were stimulated by muscarine. Nicotinic receptor activation increased the phosphorylation of Ser-19, Ser-31, and Ser-40 on tyrosine hydroxylase, and this was inhibited by clonidine in a concentration-dependent manner. On the other hand, clonidine had no effect on calcium uptake, yet increased the phosphorylation of Ser-19 under basal conditions. Using calcium and calmodulin-stimulated protein kinase II obtained from rat brain clonidine increased the autophosphorylation of the α-subunit of the kinase by 37%, and also its activity against an exogenous peptide substrate by 29%. These data are consistent with the hypothesis that clonidine inhibits nicotinic receptor-induced tyrosine hydroxylase phosphorylation by decreasing calcium influx into chromaffin cells, perhaps by an action at the nicotinic receptor. Clonidine also increases the basal phosphorylation of tyrosine hydroxylase at Ser-19, perhaps by directly activating calcium and calmodulin-stimulated protein kinase II. BIOCHEM PHARMACOL 51;3:239–245, 1996.

KEY WORDS. clonidine; nicotine; tyrosine hydroxylase; phosphorylation; protein kinases; noradrenaline release; calcium uptake; chromaffin cell

The bovine adrenal medullary chromaffin cell is a useful model for studying mechanisms controlling the release and synthesis of catecholamines. Stimulation with acetylcholine via nicotinic receptors ultimately causes voltage-sensitive Ca^{2+}† channels to open and allows the influx of extracellular Ca^{2+}, which causes release of catecholamines [1–4]. Concomitant increases occur in the phosphorylation of TOH [5–7] and in catecholamine synthesis [8, 9]. These effects can be mimicked by nicotine or direct depolarisation with K^+. Acetylcholine also activates muscarinic receptors, and stimulation of chromaffin cells with muscarine increases TOH phosphorylation [7], but causes little or no catecholamine synthesis [10] or release [2]. Muscarine stimulates an increase in cyclic-GMP levels, and this has been linked with a decrease, an increase, or no change in catecholamine release induced by nicotine [11–13].

Whereas the mechanisms involved in catecholamine release following the entry of extracellular Ca^{2+} are not entirely established [2], the mechanisms leading to phosphorylation of TOH and the increase in catecholamine synthesis are quite well understood. TOH is the rate-limiting enzyme in the synthesis of catecholamines, and phosphorylation increases its enzymic activity [8, 14]. TOH is phosphorylated on Ser-8, Ser-19, Ser-31, and Ser-40 by a number of protein kinases in intact chromaffin cells, including those regulated by Ca^{2+}, diacylglycerol, cyclic-AMP, and cyclic-GMP [7, 15–17]. It is proposed that the influx of extracellular Ca^{2+} induced by acetylcholine, nicotine, or K^+ initially increases the phosphorylation of Ser-19 by activation of Ca^{2+} and calmodulin-stimulated protein kinase II (CaM-PK II); then Ser-40 is phosphorylated by activation of protein kinase A (PKA), and later Ser-31 is phosphorylated by activation of protein kinase C (PKC) and/or the extracellular receptor kinase (ERK) [5, 6, 7]. Ser-8 is phosphorylated by a proline-directed protein kinase, but is unaffected by depolarisation or second messenger activation in chromaffin cells [7]. Muscarine mobilises intracellular Ca^{2+} and increases TOH phosphorylation via activation of PKC and/or protein kinase G (PKG) [7, 17].

It has been shown previously [18, 19] that clonidine inhibits the release of catecholamines evoked by acetylcholine and nicotine. However, clonidine did not inhibit the release evoked by veratridine, K^+, barium, or histamine [18, 20]. This

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† *Abbreviations:* TOH, tyrosine hydroxylase; NA, noradrenaline, PAGE, polyacrylamide gel electrophoresis; Ca^{2+}, calcium; CaM-PK II, calcium and calmodulin stimulated protein kinase II; PKC, protein kinase C; PKA, protein kinase A; ERK, extracellular receptor kinase; HBLS, Hepes buffered Lockes solution.

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specificity, the lack of effect of other α-adrenoceptor agonists, and the failure of α-adrenoceptor antagonists to reduce the effect of clonidine on nicotine-evoked catecholamine release suggested that clonidine may act not through α-adrenoceptors, but rather at the nicotinic receptor itself, or its associated ion channel, to decrease the initial depolarisation of the cell [18]. Yanighara *et al.* [9] found that clonidine inhibited the carbachol-stimulated synthesis of catecholamines and tyrosine hydroxylase activation in adrenal chromaffin cells and provided evidence that this was due to inhibition of the carbachol-stimulated uptake of Ca^{2+}. They also found that the α_2-adrenoceptor antagonist yohimbine failed to antagonize the inhibitory effects of clonidine. Adrenal chromaffin cells contain an imidazoline binding site that binds clonidine [21]. Regunathan *et al.* [22] had found that clonidine, and other imidazoline receptor agonists, increased Ca^{2+} uptake into chromaffin cells under basal conditions, apparently "through an ion regulating system that may be different from Ca^{2+} channels". Yanighara *et al.* [9] found no effect of clonidine on calcium uptake under basal conditions. Clonidine, but not other imidazoline binding site agonists, also increased the level of cyclic-GMP in chromaffin cells apparently by uptake of clonidine into the cell and subsequent direct inhibition of a cyclic-GMP phosphodiesterase. This would be expected to increase TOH phosphorylation [7, 17]. Yanighara found that clonidine had no effect on TOH activity under basal conditions. Clonidine does not modulate the levels of cyclic-GMP, cyclic-AMP or phosphoinositides in adrenal chromaffin cells via an α_2-adrenoceptor mediated mechanism [22].

Clonidine, therefore, has multiple actions in bovine adrenal chromaffin cells mediated by more than one mechanism, and investigators had observed different effects of clonidine under basal and stimulated conditions. Under basal conditions there were differences in the observed effects of clonidine on calcium uptake, and one would predict different effects of clonidine on TOH phosphorylation from the published data [9, 22]. The aim of this study was, therefore, to determine under similar experimental conditions the effect of clonidine on the uptake of calcium and on the phosphorylation of individual sites on TOH in bovine adrenal chromaffin cells. Acetylcholine activates chromaffin cells *in vivo*, but because it acts at both nicotinic and muscarinic receptors, nicotine and muscarine were also tested separately. K^+ depolarisation was used to cause direct opening of voltage-sensitive Ca^{2+} channels.

MATERIALS AND METHODS

Chromaffin Cell Preparation

Chromaffin cells were isolated from bovine adrenal glands [23]. Percoll density gradient purified cells were plated on collagen-coated multiwell tissue culture plates (3.5 cm diameter × 1.0 cm deep) at a density of 0.5–1 × 10^6 cells/mL and maintained in Dulbecco's Modified Eagle's Medium supplemented as described elsewhere [5]. After 3–10 days in culture, the medium was removed and the cells were washed twice with warm (37°C) HBLS containing (nM): NaCl, 154; KCl, 5.2; $MgSO_4$, 1.2; $CaCl_2$, 2.2; glucose, 5.5; and Hepes 10; adjusted to pH 7.2 with NaOH.

Ca^{2+} Uptake

For Ca^{2+}-uptake experiments, washed chromaffin cells were pretreated with HBLS solution for 20 min. When appropriate, clonidine was included for the last 15 min of this preincubation period. The solution was then replaced with HBLS containing acetylcholine, nicotine, muscarine, or high K^+ solution containing 3 μCi $^{45}Ca^{2+}$. After 3 min the cells were rapidly washed 6 times (total 30 sec) by repeatedly replacing the solution with an ice-cold Ca^{2+}-free HBLS solution containing 2 mM EGTA. Cells were disrupted with 0.4N perchloric acid, supernatants were prepared by centrifugation and their $^{45}Ca^{2+}$ content was measured [25]. Each experiment documented in Table 1 was from a separate experiment using a separate cell culture. For each cell culture, the result was the mean of 2 or 3 separate wells.

Protein Phosphorylation in Intact Cells

For protein phosphorylation experiments the washed cells were preloaded for 45 min at 37°C with 1 mL HBLS containing 50 μCi/mL ^{32}P [5]. The solution was removed and replaced with either fresh HBLS or HBLS containing clonidine (10 μM) with ^{32}P, for a further 15 min. For stimulation, the solution was removed and replaced with HBLS containing acetylcholine, nicotine, muscarine, or high K^+, with or without clonidine, and again containing ^{32}P. After 3 min the phosphorylation reaction was stopped, the cells collected, and phosphoproteins fractionated by PAGE, detected by autoradiography, and levels of phosphorylation quantitated by densitometry using a Helena Scanning Densitometer [5].

TABLE 1. The effects of clonidine on Ca^{2+} uptake into bovine adrenal chromaffin cells

	n	− Clonidine	+ Clonidine	% Change	*t*	*P*
Basal	14	94.0 ± 5.2	109.6 ± 11.2	+17	0.83	NS
Acetylcholine	14	235.7 ± 14.7	143.5 ± 30.7	−69#	2.99	<0.025
Nicotine	15	454.8 ± 15.8	328.3 ± 30.9	−29#	4.10	<0.01
High K^+	15	360.6 ± 20.3	318.9 ± 21.7	−12#	1.91	NS

Ca^{2+}-uptake results are expressed as pmolCa^{2+} taken up/min, mean ± SEM. Uptake of Ca^{2+} was measured in the absence of a stimulus (basal), or after treatment with acetylcholine (50 μM), nicotine (50 μM), or high K^+ buffer (56 mM) for 3 min. Clonidine (10 μM) was added for 15 min prior to initiation of Ca^{2+} uptake #. % change values were calculated after subtraction of appropriate basal results. Student's *t*-test for pairs of values (i.e. + vs − clonidine) was used for statistical analysis, and $P < 0.05$ was considered significant.

HPLC Chromatography and Phosphopeptide Analysis

For experiments where TOH phosphorylation sites were to be analysed by HPLC chromatography, more concentrated and highly phosphorylated samples of TOH were required, and the above procedure was adjusted as follows: Samples were used at a density of 2 million cells/mL, and these were exposed to 200 μCi/ml ^{32}P for 3 hr. The protein was subsequently applied to PAGE at a higher protein concentration [6]. The TOH band was identified after autoradiography, digested, and eluted with trypsin. Then the peptides were dried, applied to a C18 reverse phase HPLC column in 0.1% trifluoroacetic acid, and eluted with a 1–15% acetonitrile in 0.1% trifluoroacetic acid gradient [6, 7, 26]. Phosphopeptides were detected using an online radioactive monitor, and each was identified as corresponding to particular sites on TOH both by its mobility on the HPLC column [7] and by the effect of agents such as phorbol esters, forskolin, and nicotine on the extent of its phosphorylation in intact cells [6, 26]. When data was analysed statistically, each separate experiment was from a separate cell culture.

Protein Phosphorylation and Dephosphorylation in Lysed Cells

Washed adrenal chromaffin cells were lysed by addition of buffer (600 μL per well) containing (final mM) Tris (pH 7.4), 30; dithiothreitol, 1; EGTA, 5; and leupeptin (5 μg/mL). The cells were scraped and homogenised, and the suspension was adjusted to 2.5 mg protein/mL with lysis buffer. A sample of the homogenate was phosphorylated, in the presence or absence of 10 μM clonidine, using the ATP-labelling procedure described by Dunkley *et al.* [26; protocol 1]. The effect of clonidine on protein phosphatases I and 2A in adrenal chromaffin cell homogenates was determined as described by Dunkley *et al.* [26; protocol 7].

CaM-PK II Activity and Autophosphorylation

Rat brain CaM-PK II was partially purified to the stage of its elution from calmodulin-Sepharose [27]. The effect of clonidine on the activity of CaM-PK II against an exogenous peptide substrate or the extent of autophosphorylation of CaM-PK II was estimated as described by Jarvie and Dunkley [28; sections 3.6 and 3.4, respectively].

Statistical Analysis

Student's *t*-test was used for all statistical analysis; $P < 0.05$ was considered to reflect a significant difference. For the peptide phosphorylation data, logarithmic transformations were undertaken prior to the application of the *t*-test.

Materials

Clonidine (pure substance) was a gift from Boehringer Ingelheim. ^{45}Ca (specific activity 10–40 mCi/mg), $^{32}P_i$ (specific activity 8500–9120 Ci/mmol) and [γ-^{32}P]ATP (specific activity >3000 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, U.K. The calmodulin-dependent protein kinase substrate was obtained from Auspep (Melbourne Cat. No. 2025). All inorganic chemicals were of analytical grade obtained either from BDH Chemicals (Australia) or from Sigma (St. Louis, MO, U.S.A.); organic chemicals were obtained from Sigma. Enzymes for digestion of adrenal medullae were obtained from Boehringer Mannheim. Materials for tissue culture were obtained from Cytosystems (NSW, Australia) or from Flow Laboratories Australasia (NSW, Australia). Percoll was obtained from Pharmacia (NSW, Australia). All drugs and chemicals were dissolved in deionized water. Final dilutions were made in HBLS.

RESULTS

$^{45}Ca^{2+}$ Uptake

The effect of acetylcholine, nicotine, and K^+ on the uptake of $^{45}Ca^{2+}$ was investigated in the presence and absence of clonidine (Table 1). Acetylcholine significantly increased the uptake of $^{45}Ca^{2+}$ above basal levels. Clonidine inhibited, by 69%, the uptake of $^{45}Ca^{2+}$ induced by acetylcholine. Nicotine increased $^{45}Ca^{2+}$ uptake to a greater extent then did acetylcholine, and clonidine significantly inhibited, by 29%, nicotine-evoked uptake. High K^+ also increased $^{45}Ca^{2+}$ uptake, but clonidine had no significant inhibitory effect. Clonidine itself caused a small but not significant increase in $^{45}Ca^{2+}$ uptake. Muscarine failed to increase $^{45}Ca^{2+}$ uptake (not shown).

Protein Phosphorylation: Intact Cells

Incubation of adrenal chromaffin cells with $^{32}P_i$ led to the phosphorylation of a number of proteins (Fig. 1, track 1). Addition of acetylcholine (25–100 μM) increased the phosphorylation of P63 (Fig. 1, tracks 7, 9, and 11), whereas 1–5 μM acetylcholine was ineffective (Fig. 1, tracks 3 and 5). P63 has previously been identified by a number of criteria as TOH [5]. The increase in TOH phosphorylation induced by acetylcholine was attenuated by clonidine (10 μM) (Fig. 1, tracks 8, 10, and 12). The increase in TOH phosphorylation with acetylcholine (25 μM) in the absence of clonidine was 124 ± 4% ($n = 3$) above basal and, in the presence of clonidine, was reduced to 110 ± 5% above basal. The inhibition by clonidine was significant ($P < 0.05$). A similar percentage reduction of clonidine-induced inhibition was found with 50 and 100 μM acetylcholine.

Depolarisation of cells with K^+ (56 mM) or nicotine (50 μM) markedly increased the phosphorylation of P63 (Fig. 2, tracks 3 and 7). Muscarine (50 μM) led to a much smaller increase in the phosphorylation of TOH (Fig. 2, track 5). The increase in TOH phosphorylation induced by nicotine was attenuated by clonidine (Fig. 2, track 8), and this was observed in every experiment. The increase in TOH phosphorylation with nicotine in the absence of clonidine was 164 ± 5% ($n = 4$) above basal, and in the presence of clonidine was reduced to 111 ± 3% above basal. The inhibition by clonidine was significant ($P < 0.025$). Similar clonidine-induced inhibition was found with 5 μM nicotine (not shown). Basal phosphor-

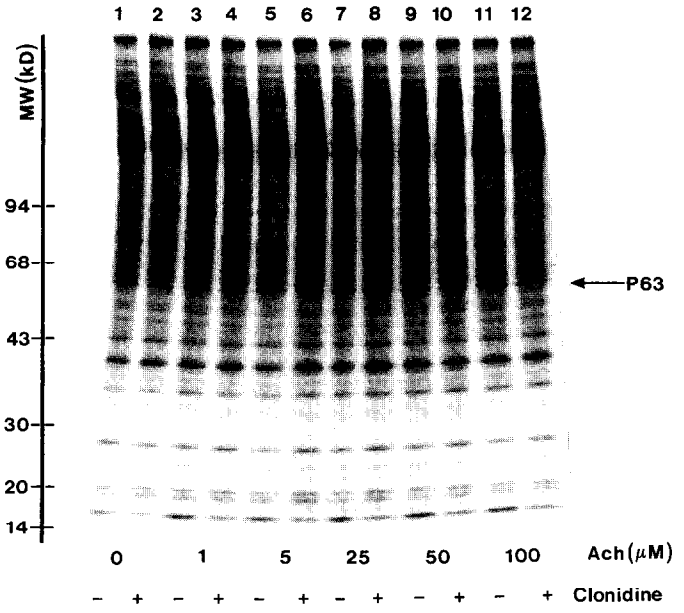

FIG. 1. The effects of clonidine (10 μM) on protein phosphorylation in intact bovine adrenal chromaffin cells. Phosphorylation was induced by stimulation with acetylcholine (Ach). Stimulation was for 3 min, after which samples were solubilised in SDS, fractionated by PAGE, and phosphoproteins detected by autoradiography. P63 (=TOH) is arrowed.

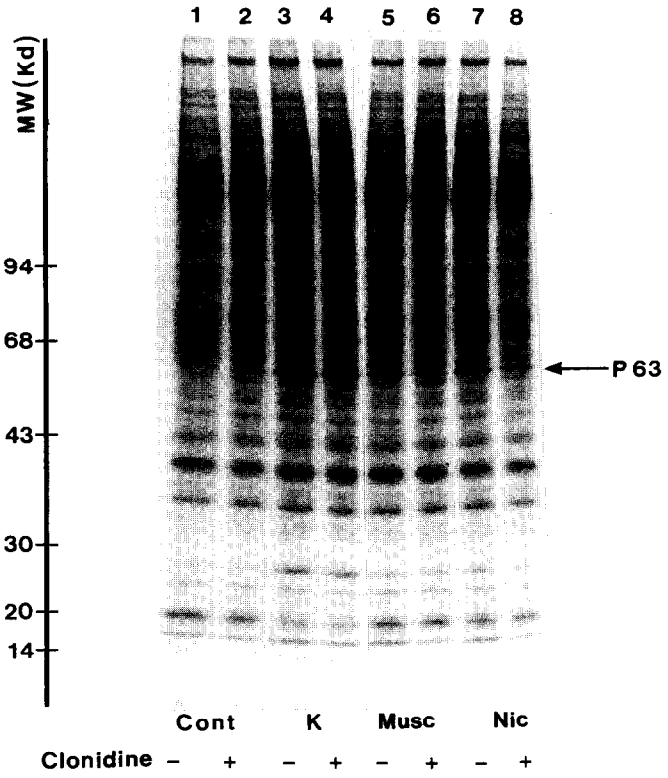

FIG. 2. The effects of clonidine (10 μM) on protein phosphorylation in intact bovine adrenal chromaffin cells. Phosphorylation was initiated without any additions (Cont), or by stimulation with nicotine (Nic; 50 μM), muscarine (Musc; 50 μM), or K^+ (K; 56 mM). Stimulation was for 3 min, after which samples were solubilised in SDS, fractionated by PAGE, and phosphoproteins detected by autoradiography. P63 (=TOH) is arrowed.

ylation of TOH was not significantly effected by clonidine, nor was TOH phosphorylation inhibited by clonidine when the cells were stimulated with either high-K^+ buffer or muscarine (Fig. 1, tracks 2, 4, and 6). This was observed in all of the experiments performed.

TOH Phosphorylation Site Analysis

Digestion of radiolabelled TOH (taken from experimental gels run as in Figs. 1 and 2) with trypsin generated a number of phosphopeptides, which, when fractionated on an HPLC column and run through an online radioisotope detector, produced a series of peaks (Fig. 3A). Peaks corresponding to Ser-8, Ser-19, Ser-31, and Ser-40 on TOH were identified [26]. It is established that two peaks of radioactivity correspond to Ser-19, and these are generated by trypsin digestion of TOH at adjacent basic residues, leading to peptides with different mobility on the reversed phase column [7]. When quantitative data was required, we summed the changes in both forms of Ser-19. The phosphorylation of the peaks corresponding to Ser-19, Ser-31, and Ser-40 on TOH was markedly increased in the presence of nicotine (compare Figs. 3A and D). Clonidine (10 μM) caused an inhibition of the nicotine-stimulated phosphorylation of all these TOH sites (compare Figs. 3D and 3E). Increasing the concentration of clonidine to 50 μM further increased the degree of inhibition of nicotine-stimulated TOH phosphorylation (Fig. 3F). Comparable results were obtained in three separate experiments. Quantitation of these experiments indicated that the nicotine-stimulated phosphorylation of Ser-19 was inhibited by 56% at 50 μM clonidine; Ser-40 and Ser-31 were inhibited by 68% and by 76%, respectively.

In contrast, under basal conditions 10 μM clonidine had no effect on the phosphorylation of any site on TOH. Increasing the concentration of clonidine to 50 μM increased the degree of phosphorylation of Ser-19 by 65% (compare Figs. 3A and 3C). The phosphorylation of Ser-31 and Ser-40 was unaltered by clonidine at any concentration under basal conditions. These effects were seen in all three experiments.

Protein Phosphorylation: Lysed Cells

Lysis of chromaffin cells and incubation of the homogenates with [γ-^{32}P]ATP led to the phosphorylation of a number of proteins including TOH (not shown). Clonidine had no reproducible effect on the phosphorylation of any of the adrenal chromaffin cell proteins under any of the conditions used. The level of protein phosphatase activity in chromaffin cell homogenates was also not affected by clonidine (not shown).

CaM-PK II Phosphorylation and Activity

Incubation of a partially purified preparation of CaM-PK II from rat brain with calcium and calmodulin led to the phos-

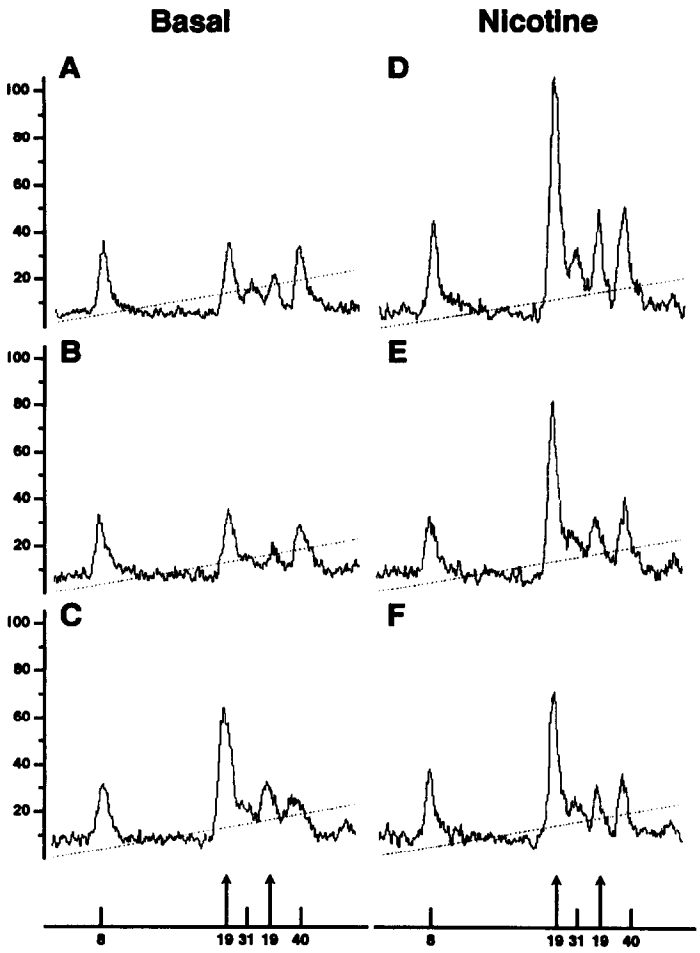

FIG. 3. The effects of clonidine (10 μM) on the phosphorylation of individual sites on TOH in intact bovine adrenal chromaffin cells. Phosphorylation was allowed to proceed under basal conditions (A–C) or was induced with nicotine (50 μM; D–F). A and D in absence of clonidine, B and E + clonidine 10 μM, C and F + clonidine 50 μM. Stimulation was for 3 min, after which samples were solubilised in SDS, fractionated by PAGE, and phosphoproteins detected by autoradiography. The TOH band was identified, digested with trypsin, and the phosphopeptides fractionated by HPLC and detected by an online radioisotope detector. Ser-40 and Ser-31 were represented by single peaks, whereas Ser-19 was represented by two peaks. The scales on the left-hand side represent the relative level of radioactive $^{32}P_i$ in each peak.

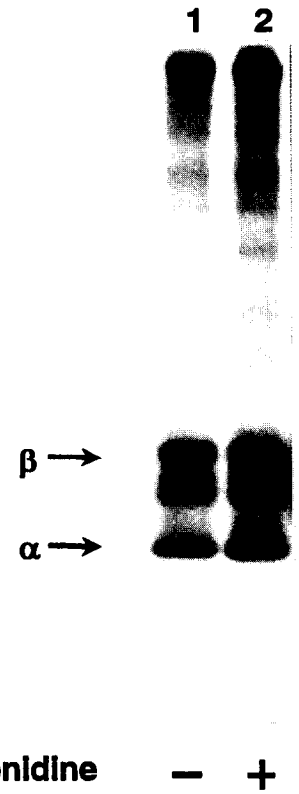

FIG. 4. The effects of clonidine (10 μM) on the autophosphorylation of CaM-PK II. Partially purified CaM-PK II from rat forebrain was incubated with [γ-^{32}P]ATP for 15 sec in the presence of Ca^{2+} (0.1 μM) plus calmodulin (50 μg/mL), the sample was fractionated by PAGE, and an autoradiograph was prepared. The position of the autophosphorylated α and β subunits of CaM-PK II are indicated.

phorylation of a number of substrate proteins, as well as the autophosphorylation of the α and β subunits of the kinase (Fig. 4, track 1). There was a consistent increase in the phosphorylation of substrates, as well as an autophosphorylation of the α and β subunits of CaM-PK II in the presence of clonidine (10 μM; Fig. 4, track 2). Quantitation of the data from five separate experiments indicated that there was a significant ($P < 0.05$) increase, of 37%, in phosphorylation of the α subunit.

In a parallel series of experiments, the ability of the partially purified rat brain CaM-PK II to phosphorylate an exogenous peptide substrate was determined. There was a significant ($P < 0.05$) increase, of 29% ($n = 5$ determination), in substrate phosphorylation in the presence of 10 μM clonidine.

DISCUSSION

Clonidine attenuates the effects of nicotine and acetylcholine, but not those of K^+, on calcium uptake into bovine adrenal chromaffin cells over a 3-min period. The data demonstrate the effectiveness of inhibition by 10 μM clonidine in chromaffin cells over the periods of stimulation chosen for the protein phosphorylation experiments. These results imply that clonidine was not affecting the voltage-sensitive Ca^{2+} channels opened by depolarisation, but that clonidine acts more directly at the nicotinic-acetylcholine receptor or its associated ion channel, as suggested by Powis and Baker [18]. Alternatively, clonidine could have an indirect inhibitory effect on the nicotinic receptor/ion channel perhaps via an imidazoline binding site. Regunathan *et al.* [22] found that 10 μM clonidine and other agents that bind to the imidazoline binding site increased the uptake of Ca^{2+} into chromaffin cells under basal conditions, with the uptake being maximal between 1–5 min. We did not observe any significant effect of clonidine on basal Ca^{2+} uptake over 3 min. Yanighara also found under basal conditions that there was no effect of clonidine on calcium uptake at concentrations up to 100 μM.

The effect of clonidine on overall TOH phosphorylation can be explained in terms of its effects on Ca^{2+} uptake insofar

as it attenuated the responses to acetylcholine and nicotine, but had no effect on the responses induced either by K^+ or muscarine. As TOH phosphorylation is dependent on Ca^{2+} [3, 4], the effect of clonidine on acetylcholine and nicotine-stimulated TOH phosphorylation was most probably the result of its inhibition of Ca^{2+} uptake. This decrease in overall phosphorylation of TOH is a likely basis for the clonidine-inhibition of carbachol-stimulated TOH activity and catecholamine synthesis observed previously by Yanighara *et al.* [9]. Analysis of the individual sites on TOH phosphorylated in response to nicotinic stimulation showed that phosphorylation of Ser-19, Ser-31, and Ser-40 were all increased. Clonidine significantly attenuated the nicotine-stimulated phosphorylation of these sites. This suggests that all of the protein kinase systems were affected by the reduction in Ca^{2+} availability due to inhibition by clonidine of nicotine-stimulated Ca^{2+} uptake. Under basal conditions, clonidine caused an increase in the phosphorylation of Ser-19, with no significant changes in the phosphorylation of Ser-31 and Ser-40. However, this required 50 μM clonidine; there was no change apparent in the overall phosphorylation of TOH, or in the phosphorylation of individual sites, in the presence of 10 μM clonidine. The increase in phosphorylation of Ser-19 was unlikely to be due to a change in calcium uptake [9], and could have been due to a number of factors. We found no evidence for an effect of clonidine on protein kinase activity in crude homogenates of adrenal chromaffin cells by analysis of the phosphorylation of individual proteins. However, clonidine was found to increase the activity and the autophosphorylation of partially purified CaM-PK II from rat brain, even at 10 μM. It is established that clonidine can enter chromaffin cells and alter phosphodiesterase activity within the cells [22]; therefore, it could be directly activating CaM-PK II to phosphorylate Ser-19 on TOH in the intact cell. The lack of effect of clonidine in the adrenal chromaffin cell homogenates may have been due to the exposure of an imidazoline binding site, causing an effective reduction in the clonidine concentration, clonidine degradation mechanisms, to an inactivation of CaM-PK II, or to an unavailability of TOH phosphorylation sites.

Regunathan *et al.* [22] showed that 10 μM clonidine had no effect on most second messenger systems, but caused an increase in cyclic-GMP levels in chromaffin cells with maximal levels reached after 15 min, the time used here for preincubation with clonidine. Increased cyclic-GMP would have been expected to increase the phosphorylation of Ser-40, but no effect of clonidine on this site was seen under basal conditions [17]. It is established that increases in phosphorylation of TOH at Ser-31 and Ser-40 occur in response to increases in intracellular Ca^{2+} [7]. Therefore, if clonidine had increased intracellular Ca^{2+}, as was found by Regunathan *et al.* [22], then it should have raised the levels of Ser-31 and Ser-40 phosphorylation, instead of having no effect on these sites, as was found here.

The data presented are consistent with the hypothesis that clonidine inhibits nicotinic receptor-induced TOH phosphorylation by decreasing Ca^{2+} uptake into the chromaffin cells, perhaps by an action at the nicotinic receptor or at an imidazoline binding site that interacts with the nicotinic receptor. The data are not consistent with the notion that clonidine increases Ca^{2+} uptake, or acts via an increase in cyclic-GMP levels. In addition, under basal conditions, higher levels of clonidine increased the phosphorylation of TOH at Ser-19, possibly by entering the cell and activating endogenous CaM-PK II directly.

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