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Interaction Between G Protein-Operated Receptors Eliciting Secretion in Rat Adrenals

A POSSIBLE ROLE OF PROTEIN KINASE C

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ABSTRACT. Catecholamine release induced by angiotensin II, histamine, bradykinin and methacholine from the rat adrenal gland perfused *in vitro* was studied under conditions in which the activity of protein kinase C (PKC) was modified. Perfusion of glands with 10 nM bradykinin abolished, in a reversible way, the secretion induced by short pulses of angiotensin II, histamine and methacholine but did not modify the release evoked by 23.6 mM KCl (high K^+). Perfusion with histamine or methacholine (30 μM) inhibited the secretion induced by the other agents by 30–50%, whereas incubation with angiotensin II (100 nM) caused little or no reduction in the release evoked by the other agents. The treatment of glands with 1 nM of the PKC activator phorbol 12,13-dibutyrate (PDBu) suppressed the responses induced by angiotensin II, histamine and methacholine, did not affect those evoked by bradykinin, and potentiated the secretion evoked by high K^+. The adenylate cyclase stimulator forskolin (1 μM) did not affect the basal secretion but strongly potentiated the release evoked by all secretagogues used, suggesting a role for protein kinase A (PKA) downstream of the receptor. The PKC inhibitor Ro-31-8220 partially reversed the inhibitory effect of bradykinin. Our results suggest that angiotensin II, histamine and muscarinic receptors share some common transduction mechanism that is regulated by PKC. PKC activity was enhanced by these agents PDBu ≫ bradykinin = histamine > methacholine = angiotensin II. Bradykinin receptor transduction does not appear to be regulated by PKC. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3:317–325, 1997.

KEY WORDS. adrenal medulla; angiotensin II; bradykinin; chromaffin cells; forskolin; histamine; muscarinic receptors; phorbol esters; PKA; PKC; secretion

The adrenal medulla releases catecholamines into the blood stream in response to both splanchnic nerves and humoral stimulation [1, 2]. Although secretion is mainly triggered by nicotinic receptor stimulation, other stimuli such as muscarine, histamine, bradykinin, angiotensin II or prostaglandins can also promote catecholamine secretion from adrenal medullary cells [3–7].

Most studies performed on stimulus-secretion coupling of the mentioned secretagogues have been performed on bovine adrenomedullary cells. However, the rat adrenal offers some advantages over its bovine counterpart in studying receptor transduction mechanisms regulating catecholamine secretion. Agents such as muscarinic agonists [8] or bradykinin [9] cause a robust secretion, allowing the study of the receptor transduction mechanism. The contribution of bigger intracellular stores or a link of these stores to the plasmalema in the rat chromaffin cell could explain the effect of substances such as histamine [10], bradykinin [6] or muscarine [11]. Although there are very few biochemical studies available in rats [12], data from bovine studies have indicated that stimuli that trigger secretion by these drugs are transduced through G-protein-mediated receptors coupled to PI† breakdown [13–16].

Activation of receptors linked to the hydrolysis of PI elicits the generation of diacylglycerol, a compound that stimulates PKC, and IP_3, which promotes the release of Ca^{2+} from intracellular stores. In spite of this link in their biosynthesis, diacylglycerol and IP_3 have considerable independence in their cellular actions: (i) there may be a temporal separation between the signals; (ii) they can also be generated by independent and unrelated sources; and (iii) their location within the cells is also different. The fact that several receptors have been described as coupled to the

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† *Abbreviations:* EC_{50}, effective concentration in 50% of experiments; HPLC, high performance liquid chromatography; PKA, protein kinase A; PKC, protein kinase C; PDBu, phorbol 12,13-dibutyrate; PI, phosphoinositide; IP_3, inositol 1,4,5-triphosphate; PMSF, phenylmethyl-sulfonyl-fluoride; TRICINE, (N-tris [hydroxymethylglycine; N-[2hydroxy-1,1-bis (hydroxymethil) ethyl]glycine); PIPES, piperazine-N,N′-bis[2-ethanosulfonic acid], 1,4-piperazidinediethanesulfonic acid; TCA, trichloroacetic acid; BSA, bovine serum albumin.

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same receptor transduction system (i.e. PI breakdown) does not mean that their stimulation will produce identical effects, and the receptors and their effector mechanisms may be downregulated differently.

A clear example of this notion comes from the regulation of PKC and its role in stimulus-secretion coupling. The first suggestion that PKC may potentiate Ca^{2+}-induced release appeared in a study by Knight and Baker [17] who described a facilitating effect of phorbol ester in electroporated bovine cells. Although the role of PKC in Ca^{2+}-induced secretion in the rat has not been studied, it is likely to work in the same way because phorbol esters potentiate nicotine-induced release [12].

However, the role of PKC in regulating receptor-mediated secretion in rat adrenal medulla is controversial. Warashina and Fujiwara [9] showed an inhibitory effect of phorbol esters in secretion elicited with muscarinic agents, whereas Malhotra *et al.* [12] found the opposite effect.

In this paper, we report the role of PKC in catecholamine release evoked by receptor-operated mechanisms compared with secretion elicited by high K^+ solutions. The main goal of this research was to test whether the activation of different G-protein-coupled receptors share the same transduction mechanism. Our data indicate that PKC might be involved in regulating stimulus–secretion coupling at, at least, two different sites: one on the receptor transduction pathway and the other on the secretory machinery. We have also found that the bradykinin transduction pathway does not appear to be regulated by PKC.

MATERIALS AND METHODS

Catecholamine Release Studies

Ninety female Sprague-Dawley rats, weighing 200–300 g, were anesthetized with 50 mg/kg sodium pentobarbitone i.p. All animal procedures were in strict accordance with the NIH Guide for the care and use of laboratory animals and were approved by the Ethical Committee of La Laguna University.

Both adrenal glands were exposed, and the adrenolumbar vein was canulated using a PE10 (Portex 800-100-100) tube. The glands were then removed and perfused retrogradely *in vitro* at 1 mL/min with a peristaltic pump with a Krebs-bicarbonate solution containing (in mM): NaCl, 119; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; and glucose, 11; pH was kept at 7.4 by continuous bubbling with 95% O_2 and 5% CO_2. The glands were cleaned of the surrounding fat, and several punctures were made with a 25G needle to facilitate the efflux of perfusate. All secretion experiments were performed at room temperature (22–24°C).

Electronically driven, three-way valves (General Valve Corp., Fairfield, NJ, USA) were placed close to the entry of the peristaltic pump to apply the drug with precision and to reduce the dead space.

Catecholamine detection was carried out as described by Borges *et al.* [18] but adapted to the rat. In brief, glands were placed in individual hermetic plastic chambers, and the perfusate was passed through an LC4B electrochemical detector obtained from Bioanalytical Systems Inc. (West Lafayette, IN, USA). An oxidation potential of +650 mV was maintained between a glassy carbon working electrode and an Ag/AgCl reference electrode. The oxidation current was recorded continuously on an ABB SE110 chart recorder. At the end of the experiments, adrenaline and noradrenaline standards were passed through the detector cell to calibrate the oxidation currents caused by secreted catecholamines. Oxidation currents had a linear relation to catecholamine concentration in the range studied (0.1–10 μg/mL). Under these conditions, the glands maintained secretory responses for over 10 hr. No damaged or edematous cells were found on histological analyses. HPLC separation of the perfusate showed that at least 95% of the total oxidation currents were caused by catecholamines [19, 20].

Using perfused glands required many animals because the same tissue can be used only once. For this reason, it was necessary to choose the concentrations of secretagogues and the stimulation patterns to be used. In the present study, the concentrations of drugs were chosen because they produce similar secretory responses (near EC_{50}) that could be maximally potentiated or attenuated by other agents: methacholine, 30 μM; histamine, 30 μM; angiotensin II, 100 nM; bradykinin, 10 nM; and K^+, 23.6 mM. Low concentrations of PDBu (1 nM), forskolin (1 μM) and Ro-31-8220 (1–10 μM) were chosen to minimize nonspecific effects. High potassium solutions were prepared by isotonic reduction of NaCl in Krebs solution.

Although chromatographic separation of catecholamines was not performed in this study, data from our laboratory [5] and from others [11] indicate that rat adrenal glands mainly secrete adrenaline (85–90%).

Incubation Experiments

Pulses of secretagogues were applied for 15 sec every 8 min. When the secretory responses became stable, these stimuli were repeated in the presence of a second drug (30–40 min) in perfused buffer and after its withdrawal (a typical "sandwich" experimental protocol is shown in the upper panel of Fig. 6a).

PKC Activity

PKC activity was assayed according to the method of Ter-Bush and Holz [20]. Glands were stimulated for 15 sec with high potassium (35 mM) solutions to check the secretory response. Ten minutes later, glands were perfused for 30 sec with or without the secretagogue. Adrenal medullas were dissected out from the cortex and homogenized in ice-cold lysis buffer containing TRICINE, 10 mM (pH 7.2); EGTA, 1 mM; leupeptin 20 μg/mL; and PMSF, 0.3 mM. Samples were centrifuged at 1000*g* for 10 min and the supernatant further centrifuged at 150,000*g* for 15 min. Pellets were

solubilized by sonication in lysis buffer containing 0.1% Triton X-100. Samples (10 μg protein) were diluted 10-fold into a PKC assay buffer with the following final solution: potassium glutamate, 14 mM; PIPES 20 mM (pH 6.6); $MgCl_2$, 10 mM; dithiothreitol, 10 mM; BSA, 0.8 mg/mL; PMSF, 0.3 mM; leupeptin, 5 μg/mL; β-mercaptoethanol, 25 mM; Triton X-100, 0.01%; ATP, 30 μM (2×10^6 c.p.m. [γ-^{32}P] ATP); histone III, 50 μg/mL; free Ca^{2+}, 10 μM (EGTA/Ca buffers); ±phosphatidylserine, 167 μg/mL; and ±diolein, 26.7 μg/mL. The reaction was carried out at 37°C and ended by the addition of 1 mL of ice-cold TCA (25% w/v). Tubes were kept on ice for 30 min and precipitates passed through 0.45-μm filters (MultiScreen™-HA, 96-well plate; Millipore Ibérica, Madrid, Spain). Membranes were washed five times with cold TCA 5% w/v and the radioactivity remaining in filters counted. PKC activity was calculated as the difference in ^{32}P incorporated into histone in the presence and absence of added phospholipids. Data are expressed as c.p.m./μg protein.

Statistical Analyses

Secretory peak height measurements were made by subtracting the basal catecholamine release level. Data are expressed as the mean ± SEM of the secretory peak heights or as representative traces. Due to the wide variability in the secretory abilities of the glands used, all values were normalized to the four control responses measured immediately before the application of the second drug. PKC activity data analysis was performed by comparison with unstimulated glands. Statistical analyses were performed with the Dunnett and Student *t* tests; $P < 0.05$ was considered significant.

Chemicals

Ro-31-8220 was a kind gift from Dr. G. Lawton (Roche Products Ltd., Herts, UK). [γ-^{32}P] ATP was obtained from New England Nuclear (Madrid, Spain). All other drugs were purchased from Sigma Chemical Co. (Poole, Dorset, UK). All salts used in the preparation of buffers were of reagent grade.

RESULTS

Time Course of Catecholamine Release Evoked by Continuous or Short Perfusion with Agonists

The concentrations of drugs used in this study were chosen because they promote similar levels of secretion when applied in short (15 sec) pulses. Longer periods of perfusion exhibited a rapid increase in secretion followed by a rapid fall to a steady-state level that, with the exception of angiotensin II, was above predrug levels and lasted for minutes. Figure 1 shows representative traces of secretion obtained when glands were continuously perfused with indicated drugs.

Although short-pulse stimulation allows one to obtain repetitive release responses for hours, angiotensin II-evoked secretion often declined continuously, even when freshly prepared solutions were used for each stimulation.

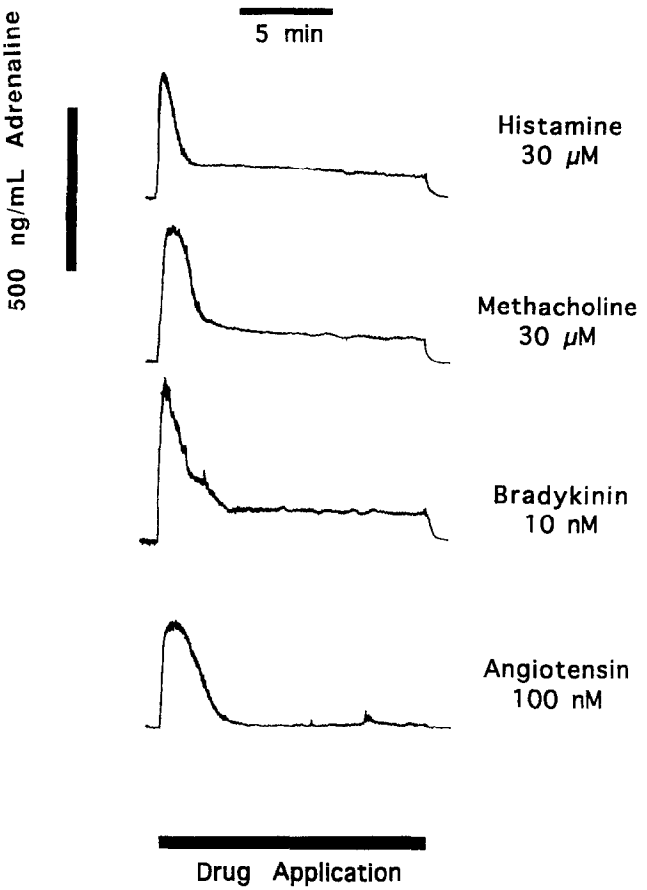

FIG. 1. Time course of catecholamine secretion evoked by different agents. Drugs, at the indicated concentrations, were perfused to the gland for 15 min, as shown by the bottom bar. The vertical bar indicates the oxidation current corresponding to that elicited by 500 ng/mL of adrenaline. Traces represent 10–12 experiments from different glands.

Effect of Secretagogue Combinations on Catecholamine Secretion

These experiments were designed to determine whether the concomitant application of agonists would facilitate or inhibit the secretory responses. Drugs were given as described in Materials and Methods (see also upper traces in Fig. 6a). Incubation of glands with 10 nM bradykinin rapidly inhibited catecholamine secretion evoked by pulses of angiotensin II, methacholine and histamine but had no effect on those evoked by high K^+. Angiotensin II caused little effect on the secretion evoked by any agent. Histamine and, to a lesser extent, methacholine inhibited the catecholamine secretion evoked with other agents by 30–50%. K^+ stimulation remained unaffected regardless of the agent (Figs. 2, 3).

The effect of the incubation with bradykinin started at the first secretory peak and was rapidly reversed after removal of the drug.

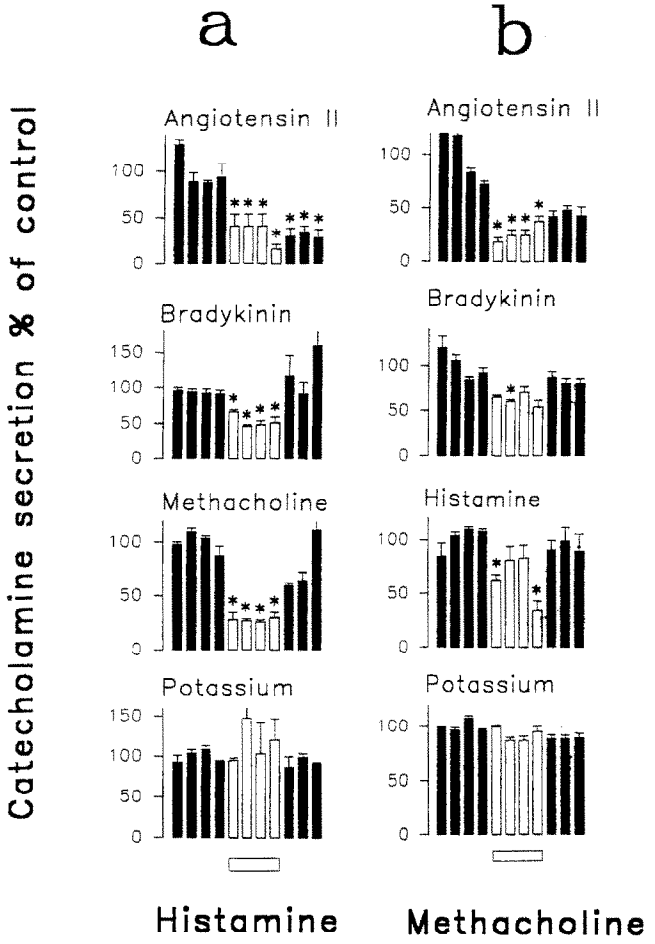

FIG. 2. Effects of histamine or methacholine incubation on catecholamine release of perfused rat adrenals. Glands were exposed to short pulses (15 sec every 8 min, as described in Materials and Methods) of the drugs indicated at the top of each graph. When the secretory responses stabilized, glands were perfused continuously for 30–40 min with 30 μM of histamine (a) or methacholine (b). Graphs show the catecholamine secretion before and after incubation (filled columns) and during incubation (open columns). Data (mean ± SEM) were obtained from the peak heights of the oxidation current subtracted from the basal release current. All data were normalized as the percentage of the average of the four pulses immediately before the second drug incubation and are from 4–12 different glands. *$P < 0.05$ compared with the average of control pulses (Dunnet test).

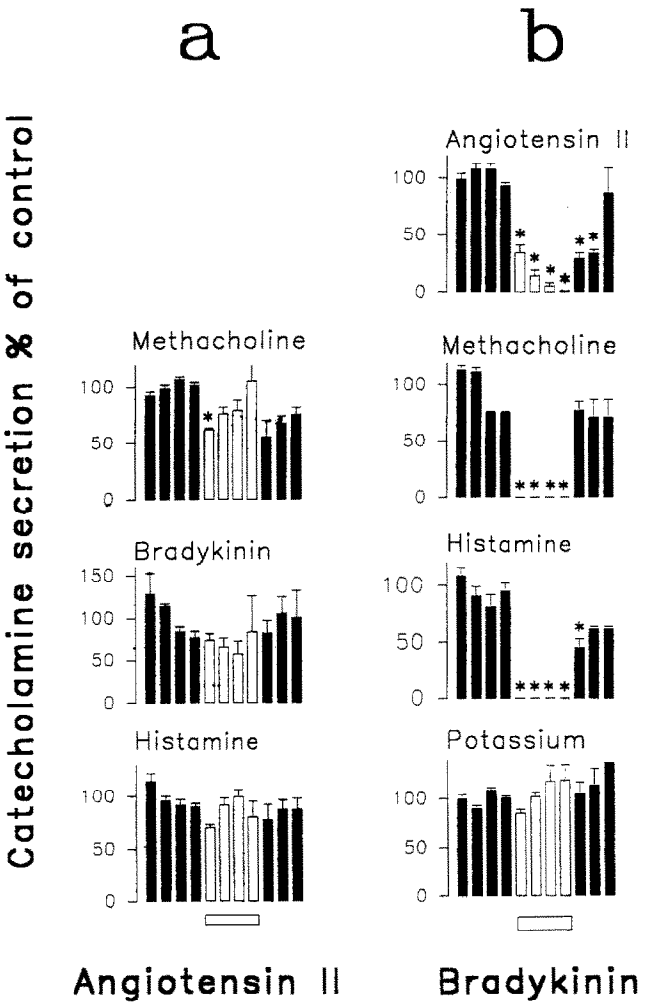

FIG. 3. Effects of angiotensin II or bradykinin incubation on catecholamine release of perfused rat adrenals. Experimental protocols and data are shown as described in Fig. 2. In this case, glands were perfused continuously with (a) angiotensin II (100 nM) or (b) bradykinin (10 nM). Data (means ± SEM) are from 4–12 different glands.

Effect of Activation and Inhibition of PKC

To explain whether cross inhibition between the secretagogues could involve PKC, a series of experiments was performed to analyze the effect of the continuous activation of PKC on stimulated catecholamine release. PDBu did not modify the basal release, although it did inhibit the secretion evoked by histamine, methacholine and angiotensin II in a time-dependent manner. However, PDBu did not affect the secretory response to bradykinin and potentiated the effect of K^+. The inhibition caused by PDBu on responses to histamine and methacholine was slowly reversed with PDBu withdrawal (Fig. 4a); responses to angiotensin II were not recovered.

Significant effects of PDBu on the secretion evoked by angiotensin II, histamine and methacholine were fully observed at a PDBu concentration of 1 nM. Higher concentrations of PDBu immediately suppressed the secretory responses to these agents in an irreversible manner (data not shown). However, the inactive phorbol ester 4-α-PMA (100 nM) failed to modify the histamine-evoked secretion (data not shown).

Experiments shown in Fig. 5 were performed to study the degree of PKC activation caused by the secretagogues and phorbol esters. PDBu (1 nM) promoted a dramatic increase in PKC activation, this effect not being observed with 4-α-PMA (100 nM; not shown). Histamine (30 μM), methacholine (30 μM), bradykinin (10 nM) and, to a lesser extent, angiotensin II (100 nM) promotes a twofold increase in the activation of PKC.

If PKC were involved in the cross talk that mediates the inhibitory effect of bradykinin, this inhibition should be at least partly blocked after inhibition of PKC. Figure 6 shows

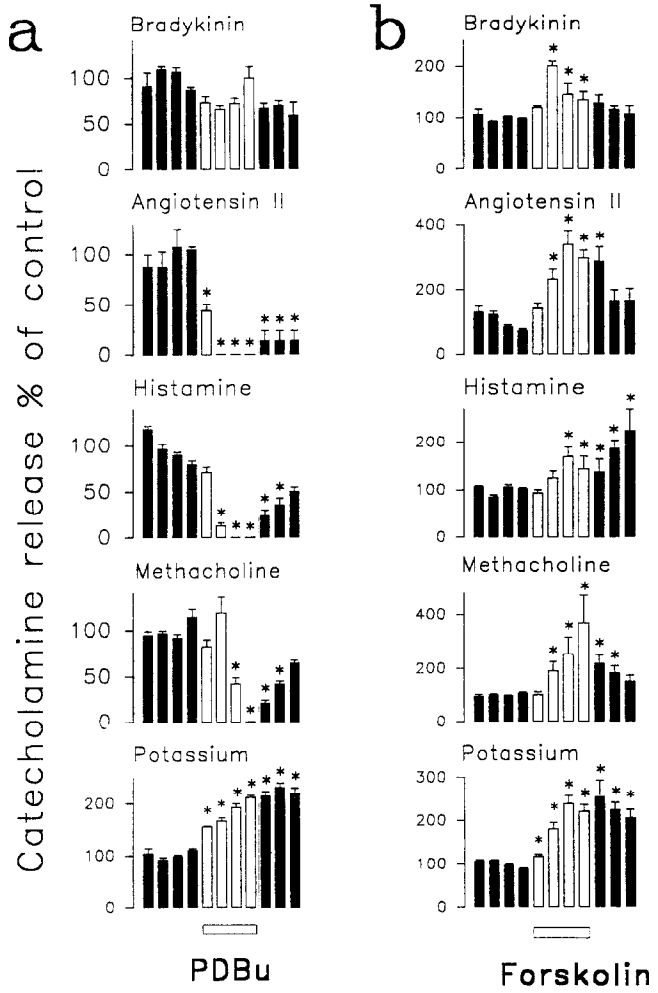

FIG. 4. Effects of PKC and PKA stimulation on catecholamine release. Experimental protocols and data are shown as described for Fig. 2. Glands were perfused with (a) forskolin (1 μM) or (b) PDBu (1 nM). Data (means ± SEM) are from 4–9 experiments with different glands. *P < 0.05 compared with control pulses.

the effect of the compound Ro-31-8220 (a highly specific PKC inhibitor) on the bradykinin–histamine interaction. Although this partial reversal of the blockade could also be observed with larger concentrations of Ro-31-8220, it also caused an increase in the basal release of catecholamines and reduced the elicited secretion caused by all agents (data not shown). Other PKC inhibitors such as H-7 and staurosporin were tested in separate experiments, but the incubation with these agents (0.1–10 μM) also suppressed the secretory responses to all secretagogues and increased the basal release.

Effect of PKA Stimulation

To test whether PKA activation could differentiate between distinct secretagogue-mediated responses, adrenal secretion was examined in the absence and presence of 1 μM of the adenylate cyclase activator forskolin. The forskolin effect was very similar on all the stimuli used. It did not

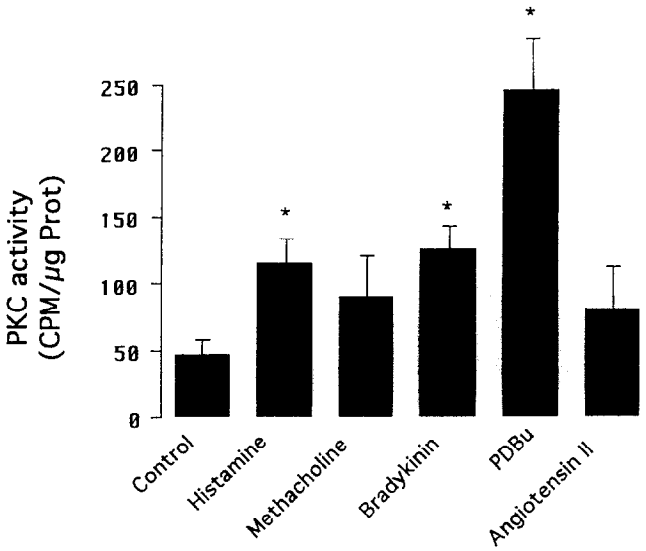

FIG. 5. Effect of different agents on PKC activity. Glands were perfused with Krebs solution containing no drugs (control) or histamine (30 μM), methacholine (30 μM), bradykinin (10 nM), angiotensin II (100 nM) or PDBu (1 nM) for 30 sec. Adrenal medullary tissues were processed as described in Materials and Methods. Experiments were performed in duplicate. Data (means ± SEM) are from 4–9 experiments with different glands. *P < 0.05 compared with control pulses.

modify the basal catecholamine release (not shown) but potentiated the secretory responses evoked by all of the secretagogues (Fig. 4b). The time courses of the secretory responses were similar for all of the stimuli used, with the exception of high K^+ and histamine, where a longer wash time was required to reverse the effect of forskolin.

DISCUSSION

Rat adrenal glands were chosen for this study because they produce healthy secretory responses to the stimulation of these receptors [5, 9, 10, 21]. In addition, whole adrenals more closely resemble physiological conditions because they are not affected by culture procedures or chronic denervation.

The role of cAMP on secretion has been the subject of controversy. Baker *et al.* [22] reported an inhibitory effect on triggered secretion, whereas Rabe *et al.*, [23] found the opposite to be true, and Knight and Baker [24] did not find any effect on permeabilized chromaffin cells.

The aim of the experiments summarized in Fig. 4b was to establish whether PKA plays a differential role in the transduction of this receptor-mediated secretion as PKC seems to do. Forskolin did not affect the basal catecholamine output but did potentiate the secretion caused by all the secretagogues tested (Fig. 4b). Our data support the view that cAMP increases elicited secretion in bovine chromaffin cells when high concentrations of the nucleotide were reached, as has been shown by Morgan *et al.* [25] who postulated that the synergistic effect of forskolin on secre-

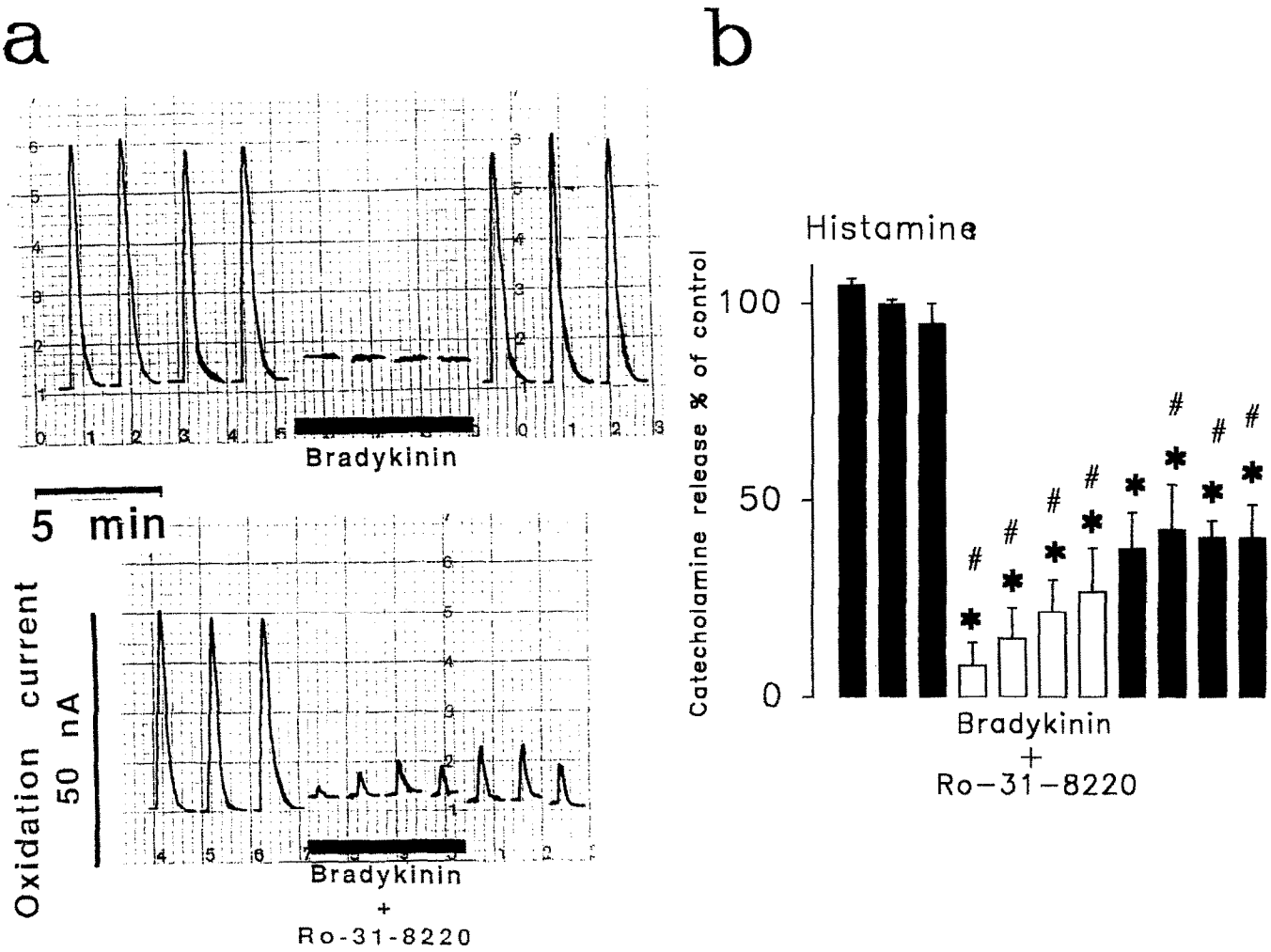

FIG. 6. Partial reversal from the inhibition of secretion caused by bradykinin after inhibition by PKC. (a) Upper traces are from a typical experiment out of four and show the effect of 10 nM of bradykinin on the secretory responses evoked by pulses of 30 μM of histamine. Lower traces show the effect of the PKC inhibitor Ro-31-8220 (1 μM) when applied with bradykinin. The vertical bar indicates the oxidation current (nA) from the secreted catecholamines. Traces are broken during the resting periods. (b) Data and Ro-31-8220 were pooled and normalized from the average of the four preceding control pulses prior to drug incubation. Filled columns indicate the catecholamine secretion obtained before and after drug incubation. Pulses of histamine were applied once the basal level reached a plateau (open columns); the initial secretion caused by bradykinin (and some times by Ro-31-8220) is not shown. Data (mean ± SEM) are from 6–8 experiments with different glands. *$P < 0.05$ compared with initial control pulses. #$P < 0.05$ compared with data from Fig. 3.

tion could be compatible with an activation of PKA, thus facilitating the exocytosis by a fairly direct action on secretory machinery. From our data, we cannot define with certainty the identity of PKA's targets on the stimulus–secretion coupling in the rat chromaffin cells, although the authors mentioned above did find that the cAMP effect is strictly Ca^{2+} dependent, which perhaps explains why basal secretion is not affected by forskolin treatment. Forskolin may also increase secretion by Ca^{2+} channel activation [26, 27], which blocks voltage-gated K^+ channels [28], and by altering the cytoskeleton organization [29].

One pharmacological approach to test whether two agents share the same mechanism of action is to study their effects alone and in combination. This approach makes it possible to check whether the interaction results in an additive or synergistic effect. Experiments shown in Figs. 2 and 3 were designed to determine whether the secretion induced by short pulses of a given secretagogue was modified by the presence of other secretagogues. Responses to stimuli that do not directly involve receptor transduction, such as depolarization with high K^+ solutions, were not affected by the continuous perfusion of glands with methacholine, histamine or bradykinin. This absence of effect was in contrast to our previous results in cat adrenal glands, where muscarinic agonists potentiated the nicotinic- and high K^+-evoked responses [8, 30], which implies important differences between these species.

Incubation with angiotensin II only slightly reduced the secretion elicited by methacholine and histamine in the first secretory peak. An explanation may be receptor de-

sensitization [31]; however, data shown in Fig. 5 show that this substance can maintain PKC activated for over 30–40 sec. Conversely, Tuominen *et al.* [32] reported that angiotensin II causes a long-term activation of PKC in bovine chromaffin cells. In the present study, continuous infusion of angiotensin II caused a transient release of catecholamine, returning rapidly to basal levels, and it was difficult to obtain consistent results with angiotensin II, even when it was applied in short pulses (Fig. 1).

The secretion evoked by histamine and methacholine appears to be mediated by a similar transduction mechanism. The secretory responses were affected by other secretagogues and by PDBu and forskolin in a similar manner (Fig. 2–4). From the results of the present study, it cannot be concluded that transduction occurs through the same or different second messenger pathways, but the identified histaminergic receptor in the rat chromaffin cell is the H_1 subtype [5], which has been coupled to inositol metabolism [32]. To our knowledge, the subtypes of muscarinic receptor in chromaffin cells have only been classified in bovine and cats [8, 30]. Although rat muscarinic receptors have not been subclassified. Malhotra *et al.* [12] found an increase in IP_3 production with muscarinic stimulation in this species.

The most surprising result of the present study comes from the experiments performed with bradykinin. Our data indicate that the bradykinin transduction pathway is regulated in a way distinct from that of the other secretagogues. We reached this conclusion based on the following observations: (i) bradykinin abolished the secretion evoked by angiotensin II, histamine and methacholine; (ii) the other secretagogues caused less modification of the catecholamine release evoked by bradykinin; and (iii) unlike the other secretagogues used, phorbol ester did not modify the secretory response to bradykinin. The last result contrasts with the data reported by Warashina and Fujiwara [9] who reported some differences in the effect of high concentrations of PDBu (250 nM) on the secretion evoked by histamine, methacholine or bradykinin and found that PDBu produced a potentiation of the release elicited by bradykinin. We cannot find an explanation for this discrepancy. In our study, a wide range of PDBu concentrations (10–300 nM) did not modify bradykinin-evoked responses, whereas these concentrations abolished, almost irreversibly, the secretory peaks evoked by the other agonists (data not shown).

Both bradykinin and PDBu incubations caused the inhibition of the secretion evoked by histamine, methacholine and angiotensin II, but the time courses of the inhibition were different. Bradykinin produced both a rapid onset and a rapid reversion, whereas PDBu required a longer time of inhibition recovery. This result can be explained by the fact that, even though both drugs act to stimulate PKC, PDBu has to pass through cell membrane to have an effect.

Phorbol ester perfusion evoked three different responses depending on the secretagogue used: it abolished muscarinic, histamine and angiotensin II responses, but did not affect bradykinin and potentiated the depolarizing stimulus of high K^+. Our results are compatible with PKC modulation of only some types of receptor transduction pathways.

PKC has at least two sites of action and one is on the secretory machinery potentiating the catecholamine release in a way similar to PKA [33], which could explain the effect of PDBu on high K^+-induced secretion (Fig. 4). This action was first described by Knight and Baker [24] in permeabilized bovine chromaffin cells and may be mediated by a PKCα subtype [34]. A second regulatory site would be in the transduction mechanism of muscarinic, angiotensin II and histamine receptors. These receptors would have a negative feedback from PKC, perhaps onto phospholipase C [16, 35], which may explain why PDBu or bradykinin activation of PKC would inhibit the transduction mechanisms of histamine, methacholine and angiotensin II. Because bradykinin-mediated secretion is not affected by PKC activation, this negative feedback is presumably not present in the transduction pathway for bradykinin receptors.

Experiments shown in Fig. 5 were conducted to test the effect of the drug used on PKC activation. It is very difficult to dissect out the medullar tissue from the cortex in the rat. Even when making a careful dissection under the microscope, it is not possible to determine the amount of active enzyme present in nonchromaffin tissues. PDBu caused an important activation of PKC that is not produced by the nonactive phorbol ester 4-α-PMA. All the secretagogues used caused an increase in PKA activation, although clear differences could not be observed among them.

The inhibitory role of PKC on the receptor transduction signal should be demonstrable by reversal with PKC inhibitors. As the experiments represented in Fig. 6 show, incubation of glands with Ro-31-8220, a highly specific PKC blocker [36], partially prevented the inhibitory effect of bradykinin on histamine-evoked release. Ro-31-8220 failed to completely block inhibition by bradykinin, although it was significantly attenuated. There are several possible explanations: (i) Ro-31-8220 has to enter the cell and its time course is slow compared with bradykinin; (ii) this compound is not as selective as expected; and (iii) Ro-31-8220 also inhibits PKC coupled to the secretory machinery. In fact, H-7 and staurosporine, less selective PKC inhibitors, reduced the secretion evoked by other secretagogues. The role of PKC in the production of IP_3 evoked by histamine and bradykinin has been studied by Boarder and Challis [16], but no correlation with secretion was made. Histamine-evoked production of IP_3 was reduced more by phorbol esters than by bradykinin.

The use of perfused rat adrenals is limited by the number of animals necessary to perform a series of experiments. The differences found between secretagogues may be explained on the basis of the problems derived from single-dose experiments. There are some arguments contrary to this assumption: (i) all the concentrations caused a similar level and time course of secretion at the pace of stimulation used; (ii) the high sensibility of histamine-, methacholine- and

angiotensin II-evoked responses to incubation with bradykinin or phorbol esters cannot be explained by the concentrations of the secretagogues used; and (iii) the time course of the inhibition caused by bradykinin and PDBu and the reversal of this effect by the PKC inhibitor strongly suggest a role for PKC in this modulation. The insensibility of bradykinin-evoked secretion to PDBu, even at large doses, points toward a different regulation of both responses. It is difficult to perform biochemical studies in the rat medulla to confirm these data because, in addition to the small amount of tissue, the adrenal cortex is sensitive to the stimulation of these drugs and it is not possible to completely dissect out the inner cortex from the medulla.

The results of the present study support a role for PKC in regulating the receptor transduction of the secretory responses mediated by histamine, angiotensin II and methacholine but not on those induced by bradykinin.

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