Liberators of NO exert a dual effect on renin secretion from isolated mouse renal juxtaglomerular cells

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Schricker, Karin, and Armin Kurtz. Liberators of NO exert a dual effect on renin secretion from isolated mouse renal juxtaglomerular cells. *Am. J. Physiol.* 265 (*Renal Fluid Electrolyte Physiol.* 34): F180–F186, 1993.—This study aimed to examine the role of nitric oxide (NO) in the regulation of renin secretion from renal juxtaglomerular (JG) cells. Using primary cultures of mouse renal JG cells, we found that sodium nitroprusside (SNP) and 3-morpholino-sydnonimin-hydrochloride (SIN-1), two structurally different liberators of NO, led to a transient inhibition during the first hour followed by a marked dose-dependent stimulation of renin secretion lasting for an additional 20 h. This stimulatory effect was blunted by methylene blue (50 μM) and was reversible within minutes after removal of the NO liberators. SNP and SIN-1 also stimulated guanylate cyclase activity in the cultures with a maximum within the first hour of incubation. Increasing intracellular guanosine 3′,5′-cyclic monophosphate levels by 8-(4-chlorophenylthio)guanosine 3′,5′-cyclic monophosphate (100 μM) or by atrial natriuretic peptide (10 nM) decreased basal renin secretion but did not inhibit the effect of SNP. The stimulatory effect of SNP was not related to adenosine 3′,5′-cyclic monophosphate levels in the JG cells and was blunted after chelation of extracellular calcium by 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid. Taken together, our findings suggest that liberators of NO have two effects on renin secretion from isolated JG cells: an inhibitory effect mediated by stimulation of soluble guanylate cyclase activity and a stimulatory effect mediated by an as yet unknown pathway that requires extracellular calcium.

endothelium-derived relaxing factor; exocytosis

THERE IS ACCUMULATING evidence that the endothelium and endothelium-derived factors may exert physiologically important functions for the regulation of renin secretion from renal juxtaglomerular (JG) cells (4). In this context, good evidence has been elaborated suggesting a stimulatory effect of prostacyclin (10) and an inhibitory effect of endothelin (19, 20, 23, 27) on renin secretion. Recent findings, moreover, suggest that the endothelium-derived relaxing factor (EDRF), which is considered to be nitric oxide (NO) (22), is also capable of modulating renin secretion (1, 7, 16, 25, 31). Indirect evidence for a relevant role of EDRF in the control of renin secretion may also arise from the observation that the enzyme NO synthase, which generates EDRF, is not only located in renal endothelial cells (18) but also in the macula densa cells that are neighbors to JG cells (24). The particular effect of EDRF/NO on renin secretion, however, is so far less clear. Studies on renal cortical slices have produced results suggesting an inhibitory effect of EDRF/NO on renin secretion (1, 31). Because EDRF/NO is known to stimulate guanylate cyclase activity in various tissues (13, 30), an inhibitory effect of EDRF/NO would be compatible with the concept that guanosine 3′,5′-cyclic monophosphate (cGMP) is an inhibitory second messenger for renin secretion (11, 15). On the other hand, there are studies done with isolated perfused rat kidneys suggesting a potent stimulatory effect of EDRF/NO on renin secretion (7, 25). A stimulatory rather than an inhibitory effect of EDRF/NO would be supported by indirect evidence obtained from coculture studies with renal JG and capillary endothelial cells (16). These opposite interpretations about the effect of EDRF/NO on renin secretion primarily obtained in complex preparations such as kidney slices or perfused kidneys prompted us to examine the effect of NO on renin secretion in a more clear model, which we considered to be isolated renal JG cells. To this end we have studied the effects of liberators of NO such as sodium nitroprusside (21) and 3-morpholino-sydnonimin-hydrochloride (SIN-1) (2) on renin secretion from primary cultures of mouse renal JG cells, which are a suitable model to study the regulation of renin secretion in vitro (5).

Our findings suggest that NO has a dual effect on renin secretion from isolated JG cells, namely a transient inhibitory effect mediated by stimulation of guanylate cyclase and a stimulatory effect mediated by an as yet unidentified pathway requiring extracellular calcium.

METHODS

Culture of mouse JG cells. Mouse JG cells were isolated as described previously (5). For one cell preparation, four male C57Bl6 mice (4–6 wk old) who had free access to normal food and water were killed by decapitation. The kidneys were extirpated, decapsulated, and minced with a razor blade. The minced tissue was incubated under gentle stirring for 70 min at 37°C in a solution of 30 ml *buffer 1* [in mM: 130 NaCl, 5 KCl, 2 $CaCl_2$, 10 glucose, 20 sucrose, and 10 tris(hydroxymethyl)aminomethane hydrochloride, pH 7.4] supplemented with 0.25% trypsin (Sigma) and 0.1% collagenase (Boehringer Mannheim).

After enzymatic dissociation, the tissue was sieved over a 22-μm screen. Single cells passing the screen were collected, washed, and resuspended in 4 ml of *buffer 1*; they were further separated using Percoll (Pharmacia) density gradients. The obtained cell suspension was added to two tubes each containing 30 ml of 30% isosmotic Percoll in *buffer 1*. After 25-min centrifugation at 4°C and 27,000 *g*, four cell layers with different specific renin activity were obtained. The cellular layer (density, 1.07 g/ml) that had the highest specific renin activity was used for cell culture.

These cells were washed in *buffer 1* and resuspended in 3 ml RPMI 1640 medium (Biochrom, Berlin, Germany) containing 0.66 U/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2% fetal calf serum (FCS). The cultures were distributed in 50-μl portions into 96-well plates and incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air.

Experiments on renin secretion. Experiments on renin secretion were started after 20 h of primary culture. At this time point, the culture medium was removed, and the cultures were washed once with 100 μl RPMI 1640 medium containing 2% FCS. Then, 50 μl of fresh and prewarmed culture medium with the chemicals to be tested were added.

Experiments on renin secretion were performed for various

times up to 24 h. At the end of the experiment, supernatants were collected and centrifuged at 500–1,000 *g* at room temperature to remove cellular debris. The supernatants were then stored at −20°C for further processing.

Cells were lysed by adding to each culture well 50 μl of phosphate-buffered saline (PBS) containing 0.1% of Triton X-100 and shaking for 45 min at room temperature. The lysed cells were stored at −20°C for further processing.

Renin secretion rates were estimated from the appearance rate of renin activity in the culture medium. To minimize differences among different cell culture preparations, renin secretion rates were calculated as fractional release of total renin [i.e., renin activity released/(renin activity released + renin activity remaining in the cells)].

Renin activity was determined by its ability to generate angiotensin I (ANG I) from the plasma of bilaterally nephrectomized rats (17). ANG I was measured by radioimmunoassay (Sorin Biomedica, Düsseldorf, Germany).

Measurement of cyclic nucleotides. For measurement of cGMP content, JG cells were grown in 24-well plates. Fifteen minutes before harvesting of cells by addition of ice-cold perchloric acid (1.1 M), phosphodiesterase activity was inhibited by 3-isobutyl-1-methylxanthine (0.5 mM). The content of cGMP was determined in succinylated samples by radioimmunoassay (Dianova, Hamburg, Germany). Levels of adenosine 3′,5′-cyclic monophosphate (cAMP) were measured in JG cells grown in 96-well plates. After the medium was removed, cAMP was extracted by incubating the cells for 20 h in 95% ethanol-20 mM HCl at −20°C. Ethanol was evaporated, and succinylated samples were analyzed by radioimmunoassay (Amersham, Braunschweig, Germany).

Assay of protein. Protein concentration in cellular lysates was determined using the Bio-Rad protein assay kit.

Chemicals. Sodium nitroprusside, forskolin, and methylene blue were purchased from Sigma International. 3-Isobutyl-1-methylxanthine was obtained from Serva (Heidelberg, Germany). 8-(4-Chlorophenylthio)guanosine 3′,5′-cyclic monophosphate (CPT-cGMP) was from Biolog (Bremen, Germany). SIN-1 was a generous gift from Dr. Henning (Casella, Darmstadt, Germany).

Statistics. Levels of significance were calculated using Student's *t* test with Bonferroni's reduction for multiple comparisons. $P < 0.05$ was considered significant.

RESULTS

For this study we used a total of 28 different cell preparations obtained from 112 mice. Total renin activity (i.e., renin activity in supernatants and cells) of the cultures after 20 h of primary culture was 12.6 ± 1.4 (SE) μg ANG I·h^{-1}·mg protein^{-1} ($n = 28$) for the different preparations and did not change during incubation with the various drugs mentioned.

To examine the effect of NO on renin secretion, we utilized established liberators of NO, such as sodium nitroprusside (SNP) (21) and SIN-1 (2). As shown in Figs. 1 and 2, SNP and SIN-1 had similar effects on renin secretion from primary cultures of mouse renal JG cells. Both drugs decreased renin secretion during the first hour of incubation (Figs. 1 and 2). After 20 h of incubation, however, a potent dose-dependent stimulatory effect of SNP and SIN-1 was apparent (Figs. 1 and 2). To obtain more information about the time dependency of the effects, we examined the effect of 100 μM SNP after different times of incubation (Fig. 3). As shown in Fig. 3, renin secretion in presence of SNP was below basal renin

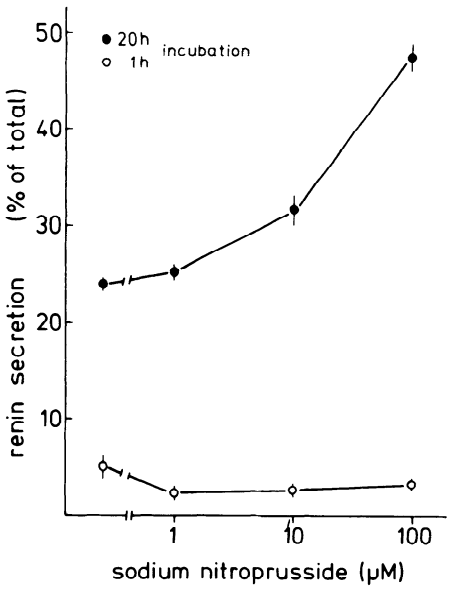

Fig. 1. Dose-dependent effect of sodium nitroprusside (SNP) on renin secretion from cultured mouse juxtaglomerular (JG) cells after 1 and 20 h of incubation. Data are means ± SE of 7 experiments.

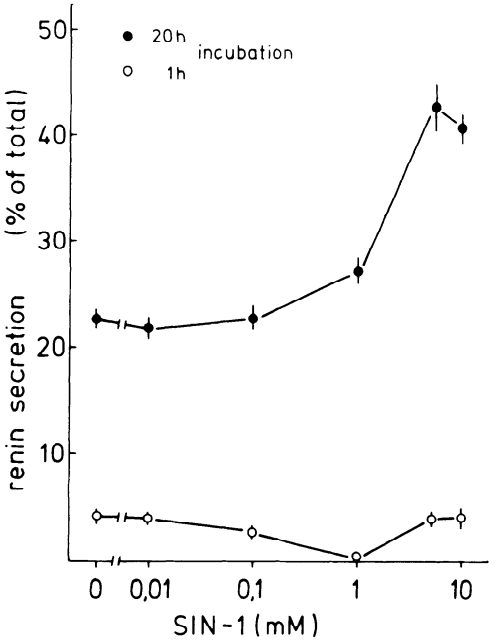

Fig. 2. Dose-dependent effect of 3-morpholino-sydnonimin-hydrochloride (SIN-1) on renin secretion from cultured mouse JG cells after 1 and 20 h of incubation. Data are means ± SE of 4 experiments.

release during the first 2 h and exceeded basal release from the third hour on for the next 20 h.

Stimulation of soluble guanylate cyclase activity and subsequent increases of intracellular cGMP levels by NO and liberators of NO is a well-known phenomenon for different tissues. It appeared of interest therefore to assess the temporal relation between the effects on soluble guanylate cyclase and on renin secretion by SNP; to this end, cellular cGMP accumulations were assayed 0.25, 1, 2, and 20 h after addition of 100 μM SNP to the cultures (Fig. 4). Stimulation of cGMP formation was maximal within the first hour of incubation and then declined

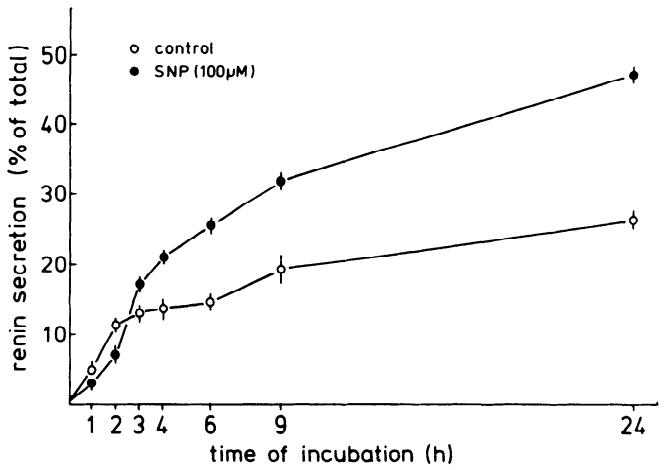

Fig. 3. Time course of renin secretion without and with SNP (100 μM). Data are means ± SE of 3 experiments.

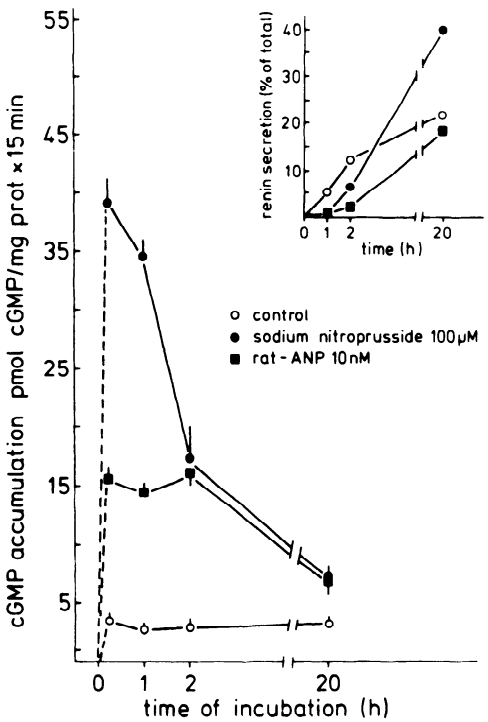

Fig. 4. Guanylate cyclase activity in cultures of mouse JG cells after different times of incubation with SNP (100 μM) and rat atrial natriuretic peptide (ANP; 10 nM). *Inset*: renin secretion rates that were determined in parallel cultures. Data are means ± SE of 4 experiments.

steadily until the 20th h of incubation, when cGMP accumulation in the presence of SNP was still twice the control value (Fig. 4). In parallel also, the effects of atrial natriuretic peptide (ANP), which is a stimulator of particulate guanylate cyclase activity in JG cells (15), were examined on renin secretion and on cGMP accumulation. As shown in Fig. 4, rat ANP (10 nM) caused a less pronounced but more constant enhancement of cGMP accumulation than did SNP. ANP led to inhibition of renin secretion during the first hour just like SNP, but in contrast to SNP, renin secretion rates remained below renin secretion rates of control cultures also for the following 20 h. Similar effects as with ANP on renin secretion were observed with the membrane-permeable cGMP analogue CPT-cGMP, a specific activator of protein kinase G (9), which also did not stimulate renin secretion during 20 h of incubation (Fig. 7). Because these findings suggested that the stimulatory effect of SNP was not causally related to cGMP formation, it was of interest to examine whether the stimulatory effect of SNP was at all directly related to the release of NO. To this end, three compounds were examined for their effects on renin secretion from isolated mouse JG cells: nitrite and nitrate, which are formed by oxidation of NO, and $Fe(CN)_6$, an Fe complex which (except for 1 more CN-group) has the same structure as the Fe complex that is left in SNP after the release of NO. As shown in Fig. 5, none of these compounds had a significant effect on renin secretion in concentrations up to 1 mM. Because it is known that NO is rapidly inactivated by methylene blue, we also examined the effect of methylene blue on the effects of SNP on renin secretion. As shown in Fig. 5, methylene blue had no effect on basal renin secretion but blunted the stimulatory effect of SNP.

In a first attempt to characterize the stimulatory effect of NO on renin secretion, we examined possible changes in cAMP levels of JG cells during 20 h of SNP incubation. Levels of cAMP increased slightly after 1-h incubation with SNP and returned to control levels within 6 h (Fig. 6). In the presence of forskolin, which causes stimulation of renin secretion comparable to SNP, cAMP levels of JG cells were increased 40-fold during the first hour and remained at an elevated level during the 20 h of incubation (Fig. 6). The difference in both the temporal pattern and the amplitude of cAMP changes in response to SNP and forskolin was taken as evidence that the SNP action is not causally related to cAMP levels. To confirm this conclusion, JG cells were incubated with SNP in the presence of forskolin. In the presence of forskolin (10 μM), renin secretion increased from 22 to 47% of total renin content. In the presence of SNP (100 μM), secretion of renin increased to 46%, and the combination of the two agents further increased the renin release to 68% of the total renin content (Fig. 7). We also examined a

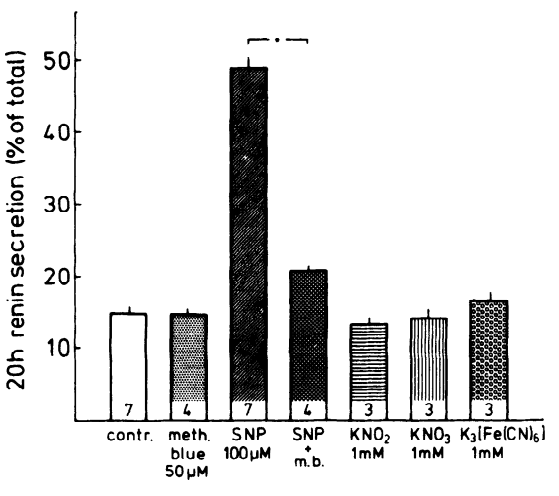

Fig. 5. Effects of KNO_2 (1 mM), KNO_3 (1 mM), and $K_3Fe(CN)_6$ (1 mM) on renin secretion from JG cells after 20 h of incubation in comparison with effect of SNP (100 μM). Also shown is effect of methylene blue (50 μM) on basal and SNP-stimulated renin secretion after 20 h. Number of experiments is indicated at bottom of bars. * $P < 0.05$.

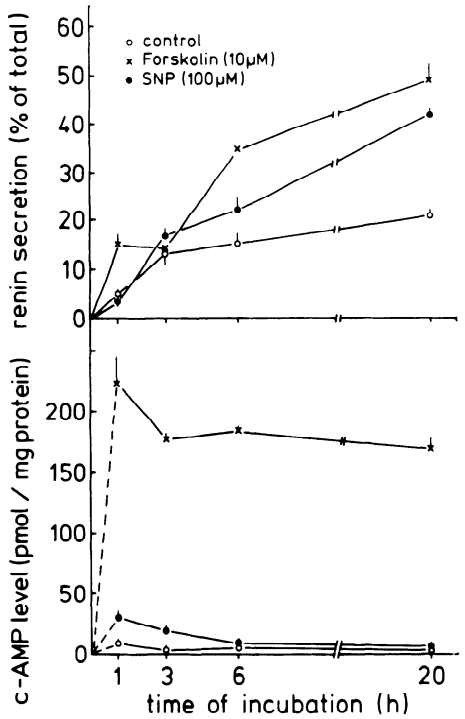

Fig. 6. *Bottom*: changes in cAMP levels of cultured JG cells during 20 h of incubation with forskolin (10 μM) and SNP (100 μM). *Top*: corresponding changes in renin secretion rates during incubation with forskolin (10 μM) and SNP (100 μM). Data are means ± SE of 4 replicate wells each.

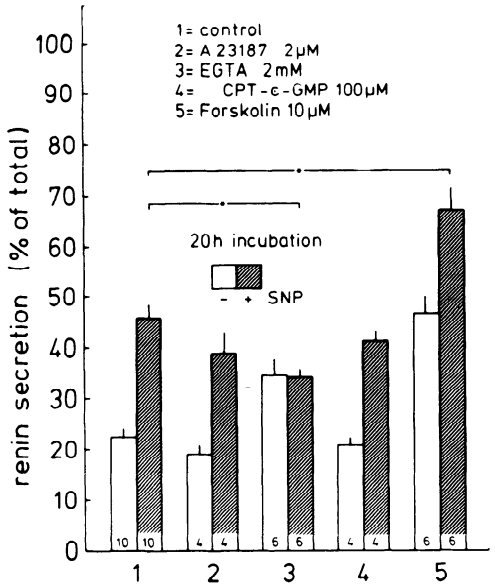

Fig. 7. Changes of spontaneous and SNP-stimulated renin secretion after 20 h of incubation with A-23187 (2 μM), EGTA (2 mM), 8-(4-chlorophenylthio)guanosine 3',5'-cyclic monophosphate (CPT-cGMP; 100 μM), and forskolin (10 μM). Addition of CPT-cGMP was repeated every 3 h during the first 12 h of incubation. Number of experiments is indicated at bottom of bars. * Significance at $P < 0.05$.

possible involvement of intracellular calcium in the action of NO on renin secretion. The Ca^{2+} ionophore A-23187 (2 μM), which was used to increase the cytosolic calcium level, did not affect either spontaneous renin secretion rate or SNP-stimulated renin secretion after 20 h of incubation (Fig. 7). On the other hand, the stimulatory effect of SNP was blunted by chelation of extracellular calcium with 2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) (Fig. 7). Addition of EGTA (2 mM) to the incubation medium alone increased basal renin secretion to 35% of total renin activity (Fig. 7).

A final set of experiments was done to examine the reversibility of the stimulatory effect of SNP (100 μM) on renin secretion. To this end, cultures established from a certain cell preparation were incubated in the presence and absence of 100 μM SNP, and renin secretion was monitored after 1, 3, 4, 6, and 24 h (Fig. 8*A*). Apparently there was a significant stimulation of renin secretion by SNP after 3 h of incubation (Fig. 8*A*). Three hours after start of the experiments, aliquots of cultures treated with or without 100 μM SNP were washed and incubated with or without 100 μM SNP, and renin secretion was monitored 1, 3, and 21 h after restart of the experiments (Fig. 8, *B* and *C*). Fractional renin secretion rates from cultures preincubated without SNP showed typical values both in the absence and in the presence of 100 μM SNP (Fig. 8*B*). A rather similar behavior was seen in cultures preincubated with 100 μM SNP for 3 h (Fig. 8*C*). Renin secretion rates after removal of SNP were similar to those measured in cultures preincubated without SNP (cf. Fig. 8*B*). Readdition of 100 μM SNP to cultures preincubated with SNP for 3 h (Fig. 8*C*) led to a temporal pattern of renin secretion similar to those treated with SNP for the first time (cf. Figs. 3 and 8, *A* and *B*).

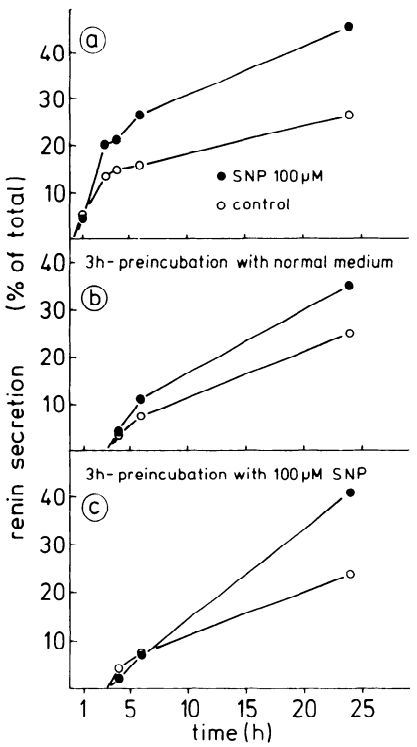

Fig. 8. Renin secretion from JG cells during 24 h of incubation without and with SNP (100 μM). *A*: time course of renin secretion without and with SNP (100 μM). *B*: same time course after a preincubation with normal medium for 3 h. *C*: same time course after a preincubation with 100 μM SNP for 3 h. Data are means ± SE of 5 replicate wells each.

DISCUSSION

This study aimed to investigate obvious contradictions concerning the effect and the role of EDRF in the control of renin secretion from renal JG cells. Today cGMP is considered to mediate essential biological effects of EDRF (13, 30). Because cGMP is an inhibitory signal for renin secretion in JG cells (11, 15), EDRF would be expected to inhibit, if anything, renin secretion. In fact, controversial interpretations about the role of EDRF on renin secretion were obtained so far (1, 7, 16, 25, 31). Although two studies suggested an inhibitory effect of EDRF on renin secretion from kidney slices (1, 31), other in vitro studies suggested a stimulatory effect of EDRF on renin secretion (7, 16, 25). The present study indicates that NO is able to exert both inhibitory and stimulatory effects on renin secretion from JG cells. The in vitro system used in our experiments was primary cultures of mouse renal JG cells in which 60–80% of cultured cells stain positive for renin (5).

To examine the effect of NO on JG cells, we used two structurally different semistable NO liberators for our prolonged cell culture studies, since free NO is rather unstable in the presence of oxygen. Both drugs, SNP and SIN-1, had similar effects on renin secretion from the cultured JG cells; they inhibited renin secretion during the first hour of incubation and stimulated renin secretion during the next hours. On a molar basis, however, SNP was more effective than SIN-1 (Figs. 1 and 2). Such a different responsiveness of cells to these two NO liberators has recently been described by Tao et al. (28). The idea that the observed effects were due to the release of NO and not to drug side effects is supported by the findings that neither the remaining substances of SNP nor the oxidation products of NO were effective on renin secretion, while rapid inactivation of NO by methylene blue abolished the effect of SNP.

We have also considered a principal problem resulting from the semipurity of the cell cultures used, namely that the stimulatory effect of NO on JG cells may be indirectly mediated by cocultured cells. To this end, the influence of the cell density of the cultures on the stimulatory effect of SNP on renin secretion was examined with the idea that such an indirect effect of SNP should be dependent on the number of cocultured cells. We found, however, that neither a threefold increase nor a threefold decrease of the normal cell density had an effect on basal or SNP- or forskolin-stimulated renin secretion, a finding that is in keeping with our previous results (16). From this finding we infer that the stimulatory effect of NO was like that of forskolin due to a direct effect on JG cells rather than to an effect mediated by cocultured cells.

Assuming that the observed effects were related to a direct effect of NO on JG cells, we have gathered more detailed information about the time dependency of NO effects on renin secretion and on stimulation of guanylate cyclase activity, which is considered as the classic second messenger pathway of EDRF/NO (13, 30). It turned out that inhibition of renin secretion by NO was associated with peak activation of guanylate cyclase activity. Enhancement of renin secretion by NO was paralleled by a decline of guanylate cyclase activity, which, however, remained significantly above the basal levels in the presence of SNP during the 20 h of incubation (Fig. 4). The temporal relationship between guanylate cyclase activity and renin secretion in the presence of SNP suggested that the inhibitory rather than the stimulatory effect was directly related to cGMP formation. This inference is supported by the observations that rising intracellular cGMP levels by ANP or a membrane-permeable cGMP analogue were inhibitory only for renin secretion (Figs. 4 and 7). An inhibitory effect of NO in short-time incubations is also in accordance with the experiments of Vidal et al. (31) and Beierwaltes and Carretero (1), who found inhibition of renin release by EDRF after 10–30 min of incubation.

In prolonged incubations, the inhibitory effect of SNP and SIN-1 changed to a stimulatory one (Figs. 1–3). This stimulatory action was independent of guanylate cyclase activity and did not require a preceding elevation of cGMP levels. Such a cGMP-independent action of SNP is already described in other tissues. For example, it had been found that NO can act as neuronal messenger in the brain without activating guanylate cyclase activity (26). In other tissues, NO was shown to modify proteins and change enzyme activity by cGMP independent ADP-ribosylation (3, 29). There is evidence that NO can exert its effects via increases of cAMP level of cells (12) or changes in intracellular calcium (8). Because cAMP and intracellular calcium are known to play a crucial role in the regulation of renin secretion (10), we examined whether the stimulatory effect of NO on JG cells is mediated by one of these second messengers. Measurement of cAMP during 20-h incubation with SNP showed a slight increase in cAMP levels of JG cells within the first hour; after the following 5 h, cAMP levels reached control values again. In contrast, forskolin, an agent which is known to exert its effect on renin secretion by cAMP, caused a 40-fold rise in cAMP levels within the first hour, and cAMP levels remained at an elevated level during the following time of incubation (Fig. 6). These different effects of SNP and forskolin on cAMP levels were a first evidence for a cAMP-independent action of SNP in JG cells. It was supported by the fact that the combined effects of SNP and forskolin on renin secretion were additive. Such an additive action is likely to result from different intracellular pathways of both agents. In a further attempt to obtain information about the action of SNP on renin secretion of JG cells, the role of calcium was examined. In BALB/c 3T3 cells it was found that NO can diminish intracellular calcium in a cGMP-independent fashion (8). Because renin secretion of JG cells is considered to be stimulated by a fall of intracellular calcium (10), an effect of NO similar to that observed in the fibroblasts could account for the stimulatory effect of NO on renin secretion. Interestingly, our findings suggest that calcium appears, in fact, to be involved in the action of NO in a way that lowering of the extracellular calcium concentration into the submicromolar range prevents the stimulatory effect of NO (Fig. 7). Lowering of the extracellular calcium concentration per se led to an increase of basal renin

secretion, an effect that has been observed in numerous experiments (for review, see Ref. 10). At which step in the intracellular events induced by NO in JG cells calcium is required, however, needs clarification in further experiments.

Apparently the calcium ionophore had no effect on SNP-stimulated renin secretion, which may indicate that normal resting cytosolic calcium concentrations are sufficient to promote a stimulation of renin secretion by SNP. Nonetheless, our finding indicates that the action of SNP cannot be explained as modulation of renin secretion according to the "calcium paradox."

Considering the characteristic time pattern of the effect of NO on renin secretion from isolated JG cells raises the question about its determinants. It appears reasonable to assume that the initial inhibition of renin secretion by NO was mediated by cGMP. Less clear, however, is the delayed appearance of the stimulatory effect, for which we wish to consider two principal reasons. First, the stimulatory effect of NO is principally stronger than the inhibitory effect mediated by cGMP, but it takes time until it is fully developed. Alternatively, the stimulatory effect of NO is of rapid onset, but it is initially suppressed by a cGMP-dependent mechanism and becomes unmasked when the inhibitory effect of cGMP runs down. The weakening of inhibition may be due to decline of guanylate cyclase activity or to other mechanisms leading to desensitization of the cGMP effect. Such a desensitization would agree with our finding that elevating intracellular cGMP levels by repeated addition of the cGMP analogue CPT-cGMP (Fig. 7) was not able to suppress stimulated renin secretion.

However, our results do not exclusively favor one of these concepts, and it may not be unlikely, therefore, that both a delayed development of the stimulatory effect as well as a rundown of the inhibitory effect of cGMP may account for the characteristic time course for the action of NO on cultured JG cells. Possible mediation of other cell types in the action of SNP probably can be excluded.

Although it will require further research to clearly elucidate the mechanism by which cGMP inhibits and NO enhances the secretion of renin in JG cells, we think that the mutual effect of NO as observed in this study could provide an explanation for the controversial interpretation about the effect of EDRF on renin secretion as derived from previous studies. Moreover, our findings also indicate that apart from cAMP, NO is another powerful stimulator of renin secretion in renal JG cells. With this stimulatory action of NO, there would exist another endothelium-derived vasorelaxant factor apart from prostacyclin (4) capable of increasing renin secretion from JG cells.

We thank R. Busse for many helpful suggestions. The expert technical and secretarial help provided by M. Hamann, K.-H. Götz, and H. Trommer is gratefully acknowledged.

This study was financially supported by Deutsche Forschungsgemeinschaft Grant Ku 859/2-1.

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Received 14 January 1993; accepted in final form 19 February 1993.

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