PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **179**, 44–49 (1985)

Cyanide and Sulfide Interact with Nitrogenous Compounds to Influence the Relaxation of Various Smooth Muscles[1] (42062)

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Abstract. Sodium nitroprusside relaxed guinea pig ileum after the segment had been submaximally contracted by either histamine or acetylcholine, intact isolated rabbit gall bladder after submaximal contraction by either acetylcholine or cholecystokinin octapeptide, and rat pulmonary artery helical strips after submaximal contraction with norepinephrine. In each of these cases the relaxation produced by nitroprusside was at least partially reversed by the subsequent addition of excess sodium cyanide. Cyanide, however, in nontoxic concentrations did not reverse the spasmolytic effects of hydroxylamine hydrochloride, sodium azide, nitroglycerin, sodium nitrite, or nitric oxide hemoglobin on guinea pig ileum, nor did cyanide alone in the same concentrations have any effect. The similar interaction between nitroprusside and cyanide on rabbit aortic strips is not dependent on the presence of an intact endothelial cell layer. Also, on rabbit aortic strips and like cyanide, sodium sulfide reversed the spasmolytic effects of azide and hydroxylamine, but it had little or no effect on the relaxation induced by papaverine. Unlike cyanide, however, sulfide augmented the relaxation induced by intraprusside, and it reversed the effects of nitric oxide hemoglobin, nitroglycerin, and nitrite. A direct chemical reaction between sulfide and nitroprusside may account for the difference between it and cyanide. Although evidence was obtained also for a direct chemical reaction between sulfide and norepinephrine, that reaction does not seem to have played a role in these results. These observations suggest the existence of at least three distinct subclasses of so-called nitric oxide vasodilators. At least in some cases cyanide and sulfide cannot be acting by the same mechanism in their modifications of the responses to the agonists.

Rabbit aortic strips contracted by norepinephrine can be relaxed by sodium nitroprusside (SNP), sodium azide (NaN_3), and hydroxylamine hydrochloride (H_2NOH). In each case this relaxation can be at least partially reversed by the addition of excess sodium cyanide (1). The inhibition of human blood platelet aggregation by the same three agents (2) is also reversed by cyanide (CN). Papaverine, sodium nitrite ($NaNO_2$), glyceryl trinitrate (GTN), and nitric oxide hemoglobin (HbNO) also relax aortic strips, but the effects of this group of agonists are not reversed by CN.

The effect of CN in these systems fulfills many of the criteria for competitive antagonism in that the antagonist, CN, has no effect of its own, the effects of both the antagonist and the agonists are reversible, the antagonist is selective for some but not all agonists and approximately parallel shifts are produced in the log dose–response curves (1, 2). In this report we show that the phenomenon is widely distributed in nature by extending demonstrations of its presence to three new organs or tissues and to two new species.

In preliminary experiments sodium sulfide (HS) appeared to mimic the effect of CN on rabbit aortic strips (1). Since that report appeared, we were made aware of a known rapid and direct chemical reaction between HS and SNP (3) which may have influenced those results. We have also found evidence for a direct chemical reaction between HS and norepinephrine. The latter does not appear to have played a role in our results, but the former may have. We have also found that HS reverses the effects of some agonists that were not reversed by CN. Moreover, under new and somewhat different conditions than those originally reported (1), sulfide appears to augment the response of aortic strips to nitroprusside.

[1] This study was supported by USPHS Grant HL 14127 from the National Heart, Lung and Blood Institute.

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0037-9727/85 $1.50

Materials and Methods. Locally obtained New Zealand white rabbits of mixed sex weighing 2 to 4 kg were sacrificed by a sharp blow to the base of the skull. The gall bladders were quickly removed and placed in iced Krebs–Ringer solution. Bile was flushed out with the same solution, and the intact bladder was suspended in a tissue bath aerated with 95% O_2 and 5% CO_2 (4). In other experiments rabbit aortic strips were prepared as previously described (1). They were used as such or the procedure of Furchgott and Zawadzki (5) was used to remove or damage the endothelial cell layer. The success of this procedure was confirmed when an added concentration of acetylcholine that produced relaxation in an intact control segment now resulted in contraction.

Guinea pigs of mixed sex weighing 450 to 500 g and male Sprague–Dawley rats weighing 300 to 400 g were obtained from Charles River Breeding Laboratories, Inc. These were housed in our animal research facility and allowed free access to food and water. They were killed by a blow to the base of the skull. Guinea pig ileum was removed as quickly as possible, flushed with Tyrode's solution, and cut into appropriate length segments for isotonic suspension in an organ bath.

Rat pulmonary arteries were cut into helical strips and suspended isometrically in the organ bath under 0.5-g tension in the same bath solution as that used with rabbit aortic strips. Cholecystokinin octapeptide was a gift from E. R. Squibb and Sons, Inc. All other chemicals were of the purest grade available commercially. Nitric oxide hemoglobin was prepared according to Kon (6). A check for complete conversion to HbNO was made by examining the visible absorption spectra (7).

Results. As shown for a representative experiment (Fig. 1), guinea pig ileum contracted by the presence of acetylcholine was immediately relaxed by the addition of SNP. In turn that effect of SNP was rapidly but only partially reversed by the subsequent addition of CN. The same was true if the segment of ileum had been contracted initially by histamine (Table I). In accord with our previous experience on rabbit aortic strips (1) CN alone in the same concentration shown in Fig. 1 had no effect on ileal segments, i.e., it neither contracted segments at rest nor relaxed previously contracted segments (not shown). If, however, the CN concentration was increased to about 5 $\times 10^{-5}$ M or higher, a slow loss of tension was observed in contracted strips. We interpret this phenomenon as representing a nonspecific poisoning of aerobic metabolism in the tissue by CN with depletion of high energy intermediates. The same observation was made in aortic strips, but at about a 10-fold higher concentration of CN (1).

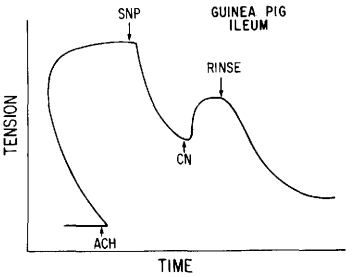

FIG. 1. A representative experiment in which a segment of guinea pig ileum was contracted by the addition of acetylcholine (ACH, 7.4 $\times 10^{-8}$ M) and subsequently relaxed by the addition of sodium nitroprusside (SNP, 2 $\times 10^{-6}$ M). The effect of SNP was rapidly reversed on the addition of sodium cyanide (CN, 7 $\times 10^{-6}$).

Unlike our experience with rabbit aorta and human blood platelets (1, 2), CN failed to reverse the effects of either NaN_3 or H_2NOH in guinea pig ileum (Table I). The failure of CN to reverse the spasmolytic effects of $NaNO_2$, GTN, or HbNO was expected since that pattern was observed also with aortic strips. It may be of significance that GTN and HbNO were at least as potent as SNP in inducing relaxation whereas $NaNO_2$, NaN_3, and H_2NOH were much weaker agonists.

As shown in Fig. 2 rabbit gall bladder contracted by the addition of cholesystokinin octapeptide (or by acetylcholine which is not shown) was relaxed by two serial additions of SNP. That effect of SNP was partially antagonized by two serial additions of CN. The effects of all these agents were reversible as shown after the rinse. As in the case of guinea pig ileum, rabbit aorta and human platelets (1, 2) CN alone had no effect.

In Fig. 3 it can be seen that a strip of rat pulmonary artery contracted by norepineph-

TABLE I. CONCENTRATIONS AT WHICH AGONISTS WERE TESTED FOR CYANIDE REVERSAL OF THEIR SPASMOLYTIC EFFECT ON GUINEA PIG ILEUM

Constricting agent	Concentration range, M	Agonist	Concentration range, M	Cyanide concentration, M	Number of animals
		Agonists reversed by cyanide			
Acetylcholine	7×10^{-8} to 7×10^{-7}	SNP	1×10^{-6} to 2×10^{-5}	7×10^{-6} to 7×10^{-5}	8
Histamine	2×10^{-5} to 3×10^{-5}	SNP	1×10^{-6} to 2×10^{-6}	7×10^{-6} to 2×10^{-5}	3
		Agonists not reversed by cyanide			
Acetylcholine	6×10^{-8} to 3×10^{-7}	$NaNO_2$	3×10^{-3} to 1×10^{-1}	1×10^{-5} to 3×10^{-5}	4
Histamine	1×10^{-5} to 2×10^{-5}	$NaNO_2$	3×10^{-3} to 5×10^{-2}	7×10^{-6} to 5×10^{-5}	3
Acetylcholine	7×10^{-8} to 7×10^{-7}	NaN_3	4×10^{-4} to 8×10^{-3}	7×10^{-6} to 2×10^{-5}	4
Histamine	1×10^{-5} to 2×10^{-5}	NaN_3	4×10^{-4} to 8×10^{-3}	7×10^{-6} to 5×10^{-5}	3
Acetylcholine	7×10^{-8} to 7×10^{-5}	NH_2OH	2×10^{-5} to 2×10^{-4}	1×10^{-5} to 4×10^{-5}	3
Histamine	1×10^{-5} to 2×10^{-5}	NH_2OH	2×10^{-4} to 8×10^{-4}	7×10^{-6} to 5×10^{-5}	3
Acetylcholine	7×10^{-8} to 4×10^{-7}	GTN	2×10^{-7} to 6×10^{-5}	1×10^{-5} to 5×10^{-5}	5
Histamine	1×10^{-5} to 2×10^{-5}	GTN	4×10^{-7} to 1×10^{-6}	2×10^{-5} to 5×10^{-5}	2
Acetylcholine	4×10^{-7} to 7×10^{-7}	HbNO	6×10^{-6} to 3×10^{-5}	7×10^{-6} to 5×10^{-5}	4

rine (NE) was relaxed on addition of SNP. That effect of SNP was also partially reversed by two serial additions of CN which alone in the same concentrations had no effect.

In experiments with rabbit aortic strips (not shown) in which the endothelial cell layer had been removed or damaged (5), the addition of acetylcholine then resulted in contraction instead of relaxation. When SNP was then added, in accord with the results of others (8), it still resulted in relaxation. That relaxation was still partially reversed by CN in concentrations of agonist and antagonist that were effective in intact aortic strips (1).

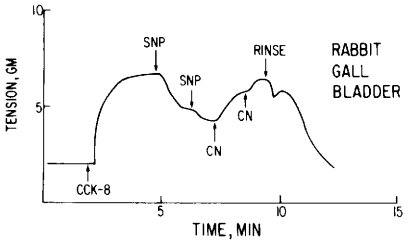

FIG. 2. A representative experiment in which a rabbit gall bladder preparation was contracted by the addition of cholecystokinin octapeptide (CCK-8, 5×10^{-9} M) and subsequently relaxed by the addition of sodium nitroprusside (SNP) in two increments, the first to 2.5×10^{-6} M and the second to a total of 7.5×10^{-6} M. The effect of SNP was reversed by the addition of two increments of sodium cyanide (CN), the first to 8×10^{-6} M and the second to a total of 4.8×10^{-5} M. As shown after the rinse the effects of all these agents were reversible.

As shown in Fig. 4, HS also produced a rapid reversal of the spasmolytic effects of NaN_3 on rabbit aortic strips contracted by NE. The same was true for H_2NOH (not shown). As also was the case with CN, HS had no effect on the relaxation induced by papaverine.

The effects of HS on the relaxation induced by SNP, GTN, HbNO, and $NaNO_2$ were all qualitatively different from those of CN. In the cases of $NaNO_2$, GTN, and HbNO, HS resulted in a reversal of the relaxation (shown for GTN only in Fig. 5). The response with GTN was somewhat unique in that it was slower in onset, and an exceptionally large excess (on the order of 1000-fold) of HS was needed. The effects of HbNO and $NaNO_2$, however, could be reversed by as little as a twofold excess of HS (not shown). In the

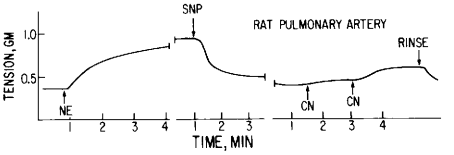

FIG. 3. A representative experiment in which a strip of rat pulmonary artery was contracted by the addition of norepinephrine (NE, 1×10^{-8} M) and subsequently relaxed by the addition of sodium nitroprusside (SNP, 1×10^{-7} M). That effect of SNP was subsequently reversed by the addition of two increments of sodium cyanide (CN) the first to 4×10^{-6} M and the second to a total of 8×10^{-6} M.

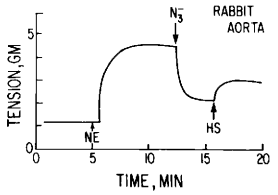

FIG. 4. A representative experiment in which a strip of rabbit aorta was contracted by norepinephrine (NE, 2×10^{-6} *M*) and subsequently relaxed by the addition of sodium azide (N_3^-, 1×10^{-6} *M*). That effect of N_3^- was partially reversed by the addition of sodium sulfide (HS, 1×10^{-4} *M*).

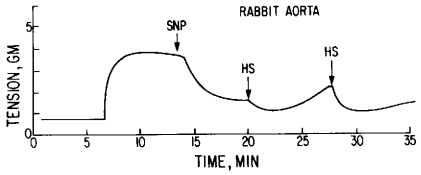

FIG. 6. A representative experiment in which a strip of rabbit aorta was contracted by norepinephrine (NE, 1×10^{-6} *M*) and subsequently relaxed by the addition of sodium nitroprusside (SNP, 2×10^{-6} *M*). The subsequent addition of sodium sulfide (HS, 1×10^{-5} *M*) resulted in an initial phase of enhanced relaxation followed by spontaneous reversal. The same was true when a second increment of HS was added to a total of 5×10^{-4} *M*.

case of SNP, the effect of HS appeared to be an augmentation of the SNP-induced relaxation (Fig. 6) instead of the anticipated reversal.

By mixing together SNP and HS in the absence of any tissue, an unstable product was obtained which after crystallization and drying at room temperature behaved exactly like SNP on the rabbit aortic strip preparation. It was about equipotent with SNP as a vasodilator, its effects were reversed by CN and augmented by HS. The visible absorption spectra matched that reported for an HS adduct to the nitrosyl group on SNP (9). A sample prepared for elemental analysis showed only approximate conformation to the elemental composition of the suspected adduct, however, probably reflecting the fact that the adduct is not only light sensitive but decomposed by aqueous solutions and heat.

In systematically checking the remaining agonists and antagonists for evidence of other possible direct chemical reactions, we noted a steady increase in absorbance with time at 280 nm when 2×10^{-6} NE (which alone has the same maximum) was mixed with 1×10^{-4} HS. The solution also became distinctly cloudy. Several experiments were then repeated using aortic strips contracted with 28 m*M* potassium instead of norepinephrine. The responses to SNP, the suspected SNP adduct, HbNO and $NaNO_2$ were observed with both CN and HS. In all cases the patterns of the responses (not shown) were the same as when NE was used to contract the strips.

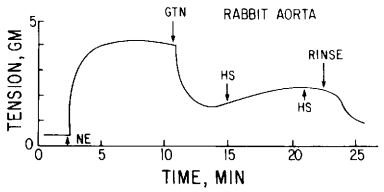

FIG. 5. A representative experiment in which a strip of rabbit aorta was contracted by norepinephrine (NE, 2×10^{-6} *M*) and subsequently relaxed by the addition of nitroglycerin (GTN, 1×10^{-7} *M*). The subsequent addition of sodium sulfide (HS, first 1×10^{-4} *M* and then up to 5×10^{-4} *M*) resulted in a reversal of the effect of GTN.

Discussion. The nature of the inferred direct chemical reaction between HS and norepinephrine is unknown. A direct addition reaction between cysteine and dopa in which the cysteine sulfhydryl group attaches to the aromatic ring when the catechol is in the form of dopaquinone has been described (10). Perhaps a similar reaction can occur with the sulfhydryl anion. In any event, two observations strongly suggest that the inferred reaction did not play a role in the results reported here. First, HS failed to reverse the effects of papaverine in norepinephrine contracted strips. Thus, HS cannot be inactivating NE when it did reverse the effects of NaN_3 or other agonists. Second, the effects of HS on SNP, SNP-HS adduct, HbNO, and $NaNO_2$ in strips contracted by 28 m*M* potassium showed exactly the same qualitative patterns as those observed in the strips contracted by NE.

We cannot, however, rule out an influence of the direct chemical reaction between HS and SNP on the observed result. Indeed, that known chemical reaction (9) probably ac-

counts for the difference between CN and HS in their interactions with SNP during relaxation of vascular smooth muscle. Our results suggest that SNP and the SNP-HS adduct act in the same way in that they are both about equipotent as agonists, and both are reversed by CN. Although these similarities may be purely coincidental, a hypothesis which also fits the observations is that SNP is not active as such, but it is activated in tissues to a sulfhydryl adduct or some unknown but similar chemical form. In terms of this hypothesis CN must either prevent such activation or in some way reverse the vasodilator action of the adduct. Therefore, this case, and this case alone, of the agonists we have examined, supports the hypothesis formulated by others (11) of the involvement of *S*-nitrosothiols as active intermediates of the "nitric oxide vasodilators."

On the basis of our previous work (1, 2), we postulated the existence of two subclasses of nitric oxide vasodilators, namely, those whose effects were, and those whose effects were not reversed by CN. In the case of the agonists where the effects were reversed by CN (NaN_3, H_2NOH, and SNP), we further postulated that CN was reversing their effects by inhibiting an unknown activator enzyme responsible for the biotransformation reaction(s) converting them to the active NO. This hypothesis was attractive because it explained why CN had no effect of its own and why CN did not reverse the effects of HbNO or NO gas. A difficulty with the hypothesis, however, was that NaN_3 and H_2NOH would require oxidative mechanisms for such a conversion whereas SNP is known to yield NO on reduction (7). The postulated activation of SNP by HS, sulfhydryl groups, or sulfane sulfur helps us out of that difficulty. Although strictly speaking it is not a chemical reduction, it is a pathway for the activation of SNP that is different from that postulated for NaN_3 and H_2NOH. Thus, our original hypothesis may be modified to suggest that both HS and CN act by blocking the activation of NaN_3 and of H_2NOH. In contrast, SNP may be activated by HS, and the effects of the SNP-HS adduct are either reversed by CN or CN prevents the adduct from exerting its vasodilator action.

With respect to vascular smooth muscle the nitric oxide vasodilators now appear to be comprised of three sub-groups: (i) NaN_3 and H_2NOH where the effects are reversed by both CN and HS, (ii) SNP where its effect is reversed by CN, but augmented by HS, and (iii) GTN, HbNO, and $NaNO_2$ where the effects are reversed by HS, but not altered by CN. At present we have no viable hypothesis about the mechanisms at work in group (iii), but we can rule out a direct chemical inactivation because HS augments platelet aggregation by GTN whereas it had no effect on aggregation induced by HbNO (unpublished observations). Others have recently suggested (12) that GTN may react directly with a ferrous heme group bound to guanylate cyclase to result ultimately in vasodilation rather than through its biotransformation to NO and an *S*-nitrosothiol.

The biochemical machinery involved in the interaction between CN and SNP appears to be widely distributed among species (human, rat, rabbit, and guinea pig) and tissues (as shown here), but the effects are most prominent in vascular smooth muscle and platelets. We have previously ruled out an involvement of prostaglandins in the platelet system (2), and observations made here would appear to rule out the participation of the so-called endothelium-derived relaxing factor (8). Perhaps the failure of CN to reverse the relaxation of guinea pig ileum by NaN_3 and H_2NOH is related to the fact that these were much weaker agonists on intestinal strips than on vascular smooth muscle or on platelets (1, 2).

Others (13) have recently reported results somewhat different from ours. Using rat thoracic aortic strips, cyanide reversed not only the effects of SNP, NaN_3, and H_2NOH, but GTN, NO gas, acetylcholine, and isoproterenol as well. With the exception of NO gas, CN also reversed the increase in c-GMP induced in the tissues by each of the agonists. In addition to the obvious species difference, these investigators used somewhat lower concentrations of NE to contract their aortic strips and the order of addition of reagents was different. In our hands (1) and those of others (14), however, the order of addition of CN vis-a-vis the agonist did not influence the ultimate response. We are at a loss to further reconcile these differences.

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Received September 28, 1984. P.S.E.B.M. 1985, Vol. 179.
Accepted January 15, 1985.