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Effects of a bicyclic thymoleptic drug (LU 3-010) on neuroeffector function in rat isolated portal veins

J. HÄGGENDAL, B. JOHANSSON, J. JONASON AND B. LJUNG

Departments of Pharmacology and Physiology, University of Göteborg, Göteborg, Sweden

The effects of the thymoleptic drug, LU 3-010, on adrenergic transmitter release and reuptake and on effector cell function in rat isolated portal veins have been investigated. The contractile response to exogenous noradrenaline was potentiated by LU 3-010 and its concentration-effect curves after LU 3-010 10^{-7} g/ml were displaced to the left to a similar extent as had been found in chronically denervated preparations, indicating efficient blockade of the membrane pump mechanism. LU 3-010 10^{-7}g/ml slightly potentiated the neurogenic response to electrical field stimulation, whereas 10^{-6} g/ml or more reduced the neural effector response in a concentration-dependent manner. The output of radioactive material per nerve impulse after incubation with ^{3}H-NA was also studied. At 10^{-7} and 3×10^{-7} g/ml of LU 3-010 no significant change in transmitter overflow was observed. At 10^{-6} g/ml there was a decrease in the "fractional release", suggesting that LU 3-010 concentrations of 10^{-6} g/ml or higher reduce transmitter release. Evidence is also given for a direct inhibitory effect of LU 3-010 on the smooth muscle.

The isolated preparation of the rat portal vein has been used to correlate transmitter release and vascular contractile response (Häggendal, Johansson & others, 1970). In studies on transmitter release it is important to quantify the amount of transmitter actively taken up by the neuronal uptake mechanism to obtain the total or "true" release. This uptake mechanism can effectively be inhibited by thymoleptic drugs, among which 3,3-dimethyl-1-(3-methylaminopropyl)-1-phenylphthalane (LU 3-010) is one of the most specific (Petersen, Lassen & others, 1966). Häggendal & others (1970) using isolated portal veins found that LU 3-010, 10^{-7} g/ml, did not increase transmitter output, whereas 10^{-6} g/ml reduced the release. This suggested that the drug might also affect the release of the transmitter as reported for other thymoleptic drugs (see e.g. Folkow, Häggendal & Lisander, 1968; Almgren, 1971). At high doses of thymoleptic drugs, an antagonism against noradrenaline has been seen and considered to be due to a blockade of adrenoceptors (cf., Haefely, Hürlimann & Thoenen, 1964). A direct inhibitory effect of desipramine on vascular smooth muscle has also been described (Hrdina & Ling, 1970). Apparently, drugs that block the neuronal uptake mechanism can exert a variety of inhibitory effects on sympathetic neuroeffector units. We have examined the influence of LU 3-010 in different concentrations on transmitter release and reuptake and on effector responses in the rat isolated portal vein.

MATERIALS AND METHODS

Mechanical recording of spontaneous activity and excitatory responses

Isolated portal vein preparations from rats of about 250 g, and of the Sprague-

Dawley strain were used. Animals were killed by a blow on the neck, the portal vein was dissected out and mounted in an organ bath filled with modified Krebs solution (for details see Axelsson, Wahlström & others, 1967). The composition of this solution was (mM): NaCl 122, KCl 4·73, $NaHCO_3$ 15·5, KH_2PO_4 1·19, $MgCl_2$ 1·19, $CaCl_2$ 2·49, glucose 11·5 and ascorbic acid 1·13. The Krebs solution was continuously bubbled with 4% carbon dioxide in oxygen at 37°. The preparation was allowed to accommodate for 1 h before the experiment was started. The contractile activity was recorded isometrically and was quantified by electronic integration. Excitatory responses were calculated as the mean force developed during a period of stimulation minus the mean force developed spontaneously during a preceding 3 min period. Responses to exogenous noradrenaline were obtained by injection of the amine into the bath for a contact time of 3 min with intervals between injections of 15 min or more, allowing complete return to spontaneous activity. Where exogenous noradrenaline was administered, continuous β-adrenoceptor blockade was maintained by propranolol 10^{-7}M for 5 min every 60 min. Neurogenic responses were induced by electrical field stimulation for 2 min (4 Hz, 0·8 ms, 15 V) (for details see Johansson, Ljung & others, 1970). Responses to increased potassium ion concentration were obtained by replacing equivalent amounts of NaCl in the Krebs solution with KCl, giving a three- or eight-fold increase in potassium ion concentration which was applied for 5 min periods.

LU 3-010 was either injected into the bath or added to the supply of Krebs solution. The preparation was exposed to a given LU 3-010 concentration for at least 18 min before any excitatory responses were induced. Effects of LU 3-010 on the spontaneous activity were measured by calculation of the mean spontaneous activity developed in the 5 min before each change in LU 3-010 concentration.

Determination of released radioactivity during electrical field stimulation

In some experiments the isolated portal veins were incubated for 30 min with (±)-[^{3}H] noradrenaline (^{3}H-NA) (10^{-7}M, specific activity 7·45 Ci/mmol, Radiochemical Centre, Amersham, England) after the 1 h accommodation period in the modified Krebs solution. After incubation, another organ bath (volume 1·5 ml) was placed in position and the portal vein was then continuously superfused at 1 ml/min with Krebs solution. In separate experiments LU 3-010 was added to the Krebs solution to give a final concentration of 10^{-7}, 3×10^{-7} or 10^{-6} g/ml. The outflow of total radioactivity was measured, the superfusates being collected as follows: during the first 30 min of washout, sampling periods of 5 min duration were used. Electrical field stimulation of the intramural adrenergic nerves (4 Hz, 0·8 ms, 15 V) was then applied for 2 min during which the superfusate was collected. After stimulation, three 5 min washout periods followed before the next stimulation began. Two such 2 min periods of nerve activity with subsequent washout phases were included in each experiment. At the end of the experiment the preparations were blotted between filter paper, weighed and homogenized in 2 ml 2N HCl by an Ultra-Turrax metal homogenizer and the tissue extracts were centrifuged and filtered. The superfusates were acidified by addition of 2 ml 2N HCl. Instagel (Packard Instrument Company) (15 ml) was added to portions of the superfusates and to the tissue extracts and the radioactivity was counted in a Packard Tri Carb Scintillation Counter. The values were corrected for efficiency and aliquot factors. The release of radioactivity induced by nerve activity was calculated as the fraction

of the mean tissue content released by each impulse (see Häggendal & others, 1970).

Determination of ^{3}H-NA and its metabolites

In some experiments the composition of the radioactivity in the superfusates was analysed. Three portal veins were mounted on the same muscle holder. The muscles were incubated with (−)-^{3}H-NA (10^{-7}M, specific activity 2·34 Ci/mmol, Radiochemical Centre, Amersham, England) and further treated as described above. In the subsequent phase of the experiment, stimulation periods of 5 min duration were used and the superfusates were collected over 5 to 10 min in flasks containing 2 ml of 2N HCl. The samples were divided into two aliquots, one for determination of noradrenaline, deaminated metabolites and deaminated 3-*O*-methylated metabolites, and the other for the determination of normetanephrine. Carrier substances were added and the samples were filtered and stored at −20° (for procedures used see Rutledge & Jonason, 1967; Jonason & Rutledge, 1968). The individual values for released noradrenaline and its catabolites in the superfusate from a stimulation period were added to the corresponding amounts present in the superfusate from the 5 min washout period after the stimulation. The figures obtained reflect the composition of the output of radioactive material during nerve activity (cf. Häggendal & others, 1970) and were expressed as a percentage of the total output.

RESULTS

Concentration-effect curves for the portal vein to exogenous noradrenaline under influence of LU 3-010 are presented in Fig. 1 (the control curve, included for comparison, was obtained under identical experimental conditions by Johansson & others, 1970). After treatment of the preparation with LU 3-010 10^{-7} g/ml, the resulting curve was markedly displaced to the left, this effect being further pronounced by LU 3-010 10^{-6} g/ml. The noradrenaline concentration giving half the maximum response (ED50) was determined graphically in each experiment and for

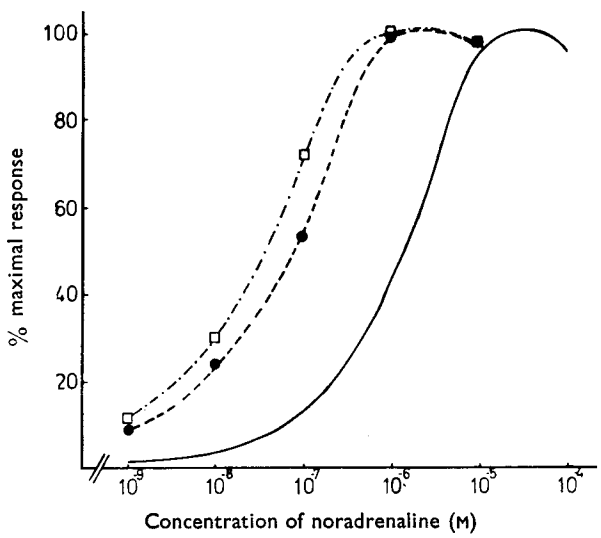

FIG. 1. Noradrenaline concentration-effect curves in normal Krebs solution and during exposure to LU 3-010 10^{-7} and 10^{-6} g/ml. Control curve taken from Johansson & others (1970). □—·—□ LU 3-010 10^{-6} g/ml (n=8); ●— —● LU 3-010 10^{-7} g/ml (n=6). — Normal Krebs solution. Each point represents the mean value for the number of rat portal vein preparations indicated.

the 15 control preparations was (12 ± 0·3) × 10^{-7}M (mean ± s.e.) (Johansson & others, 1970). After LU 3-010, 10^{-7} and 10^{-6} g/ml, the corresponding values were reduced to (1·0 ± 0·31) × 10^{-7}M and (0·40 ± 0·12) × 10^{-7}M, respectively. The values are in agreement with the capacity of LU 3-010 to efficiently inhibit the membrane pump of the adrenergic nerve terminals.

The mechanical responses obtained during repeated periods of electrical field stimulation (4 Hz) (Fig. 2) show the responses of the control preparations to be slightly reduced during the experiments. When LU 3-010, 10^{-7} g/ml, was added after the third stimulation period in one set of experiments some increase in the responses to nerve stimulation was observed compared with the untreated preparations ($P < 0·001$ in the three stimulation periods). LU 3-010, 10^{-6} g/ml, the responses to nerve stimulation ($P < 0·02$ in the last two stimulation periods).

In contrast to the clear-cut potentiation of the responses to exogenous noradrenaline seen after LU 3-010, 10^{-7} and 10^{-6} g/ml, the nerve-induced responses were slightly potentiated after concentrations of 10^{-7} g/ml and reduced after 10^{-6} g/ml. To analyse the difference between the exogenous case and nerve stimulation, the fractional release of ^{3}H-NA (see methods) was examined at different LU 3-010 concentrations. The results showed a tendency to an increased output of radioactivity after LU 3-010, 3 × 10^{-7} g/ml, from a control value of (9·76 ± 0·40) × 10^{-5} (n = 45) to (13·58 ± 1·26) × 10^{-5} (n = 4) and a reduction from this level ($P < 0·01$) is obtained after 10^{-6} g/ml to (7·50 ± 0·72) × 10^{-5} (n = 4). The composition of the released radioactivity was studied in another series of experiments and it was found that the relative amounts of noradrenaline and its metabolites in the output of radioactivity is not significantly affected by LU 3-010 treatment. In all cases noradrenaline is the predominating compound constituting more than 70% of the released material. Since there is a decrease in the output of total radioactivity after LU 3-010, 10^{-6} g/ml, the transmitter release at this drug concentration must be decreased.

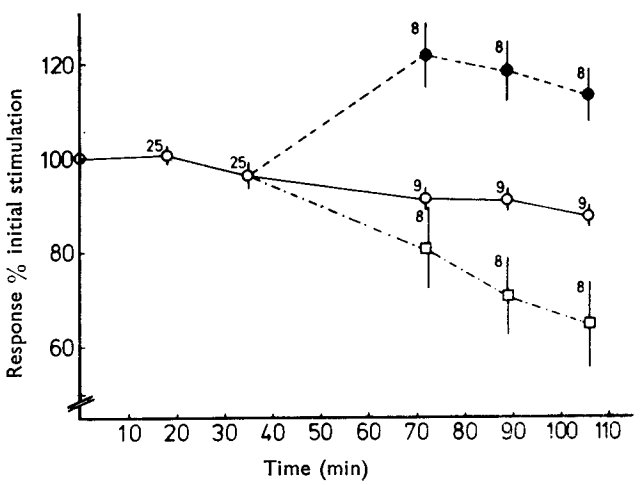

FIG. 2. Mechanical responses of the isolated rat portal vein to electrical field stimulation at 4 Hz during repeated 2 min periods. Values expressed as a percentage of the response obtained in the initial stimulation period. LU 3-010 in the concentration of 10^{-7} or 10^{-6} g/ml was administered immediately after the third stimulation period in some experiments. Each point represents the mean neurogenic response of the number of preparations indicated. Vertical lines represent s.e. ●— —● LU 3-010 10^{-7}g/ml. ○—○ Normal krebs solution. □—·—□ LU 3-010 10^{-6}g/ml.

Fig. 3 summarizes experiments designed to illustrate the effects of the drug in increasing concentrations on the response to exogenous noradrenaline (10^{-7}M), on the response to nerve stimulation (4 Hz) and on the spontaneous myogenic activity of the portal vein. The response to exogenous noradrenaline was gradually potentiated by increasing the LU 3-010 concentration from 10^{-9} to 10^{-7} g/ml. At 10^{-6} g/ml it decreased and a reduction of the response to about 45% of the control was observed at 10^{-5} g/ml. The responses to nerve stimulation were well maintained during exposure to LU 3-010 up to 10^{-7} g/ml but were decreased when higher concentrations were administered. Only about 10% of the control response was obtained by nerve stimulation at LU 3-010, 10^{-5} g/ml. The spontaneous myogenic activity was also decreased (to about 60%) at this high drug concentration but was not significantly affected at the lower concentrations.

The possibility of a direct inhibitory effect of LU 3-010 at a concentration of 10^{-5} g/ml on the smooth muscle was studied in a separate series of experiments, illustrated in Fig. 4. Contractile responses were induced by increasing the potassium

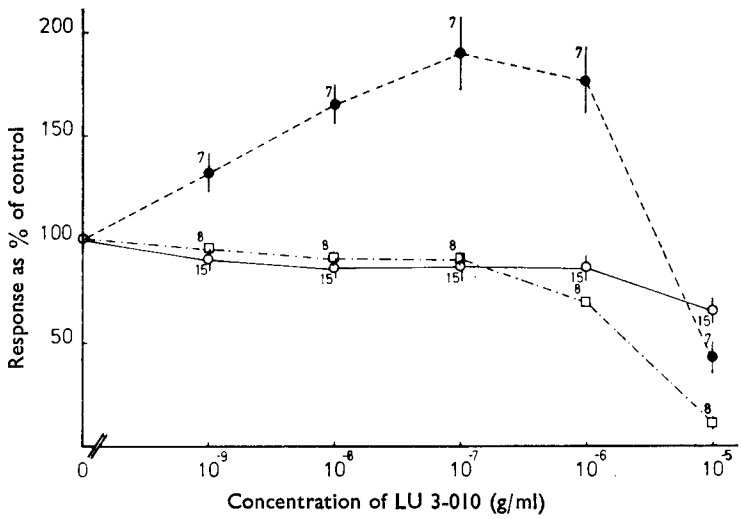

FIG. 3. Effects of LU 3-010 in different concentrations on spontaneous activity and on excitatory responses to exogenous NA (10^{-7} M) and to electrical field stimulation (4 Hz) expressed as a percentage of the individual control values obtained in normal Krebs solution. Each point represents the mean value obtained from the number of isolated rat portal veins indicated. The vertical lines represent the s.e. ○—○ spontaneous activity. □—·—□ Nerve stimulation (4Hz) ●— —● Exogenous NA 10^{-7} M.

concentration of the Krebs solution three times and eight times, by nerve stimulation and by exogenous administration of noradrenaline. In the control periods, typical intermittent spontaneous contractions of the portal vein are seen. Increase of the potassium concentration three times resulted in a phasic excitatory response, qualitatively similar to the responses to electrical field stimulation at 4 Hz and to exogenous noradrenaline. When the potassium concentration was increased eight times, a maintained smooth response was obtained. After LU 3-010, 10^{-5} g/ml, the spontaneous activity was inhibited, the amplitude of the contractions being much diminished but the contraction rate increased. Under the influence of LU 3-010, 10^{-5} g/ml, the responses to exogenous noradrenaline, to nerve stimulation at 4 Hz and to three times increased potassium concentration were changed and

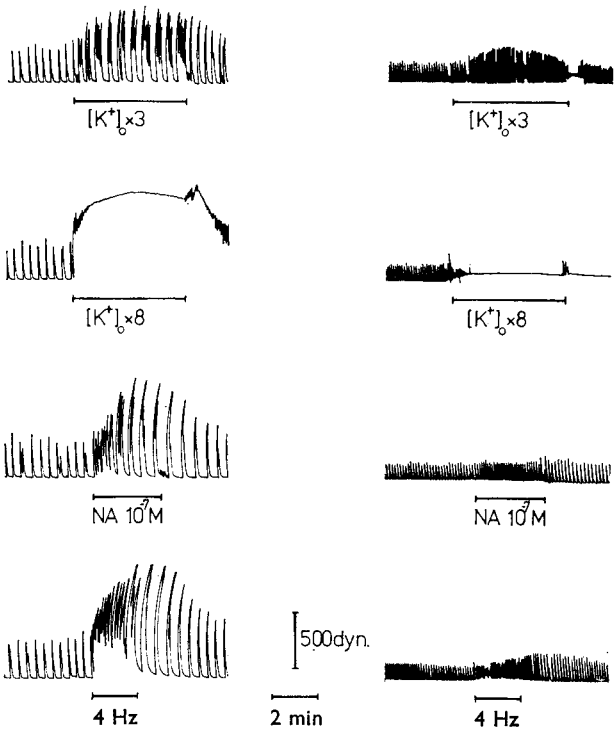

FIG. 4. Tracing from one experiment on the isolated rat portal vein. The left panel shows the isometric recording of the spontaneous activity in normal Krebs solution and of the excitatory responses to increased potassium concentrations ($[K^+]_o$), to exogenous NA 10^{-7} M and to electrical field stimulation at 4 Hz. The right panel shows the effect of LU 3-010 in a high concentration (10^{-5} g/ml) on the spontaneous activity and on the induced responses. Note the decreased amplitude of all contractions and the increased contraction rate in the presence of LU 3-010 10^{-5} g/ml.

characterized by low amplitude and high contraction rate. The smooth response to the eight-fold increase in potassium concentration was much depressed. Thus, it is evident that 10^{-5} g/ml of LU 3-010 directly interferes with the performance of the smooth muscle.

DISCUSSION

Several authors have shown that LU 3-010 is a potent inhibitor of the neuronal amine uptake mechanism located at the level of the nerve cell membrane (Petersen & others, 1966; Waldeck, 1968; Carlsson, Fuxe & others, 1969). The drug has been suggested to be among the most specific inhibitors of this uptake mechanism as it is almost devoid of anti-acetylcholine activity (see Waldeck, 1968). We found potentiation of the mechanical response to exogenous noradrenaline after LU 3-010, 10^{-9} g/ml, and at 10^{-7} g/ml the concentration-effect curve was displaced to the left to the same extent as has earlier been demonstrated to occur after elimination of the membrane pump by chronic denervation of the portal vein (Johansson & others, 1970). These findings indicate an efficient membrane pump blockade at 10^{-7} g/ml of LU 3-010. The ED50 value after LU 3-010, 10^{-6} g/ml, was reduced below that found in prejunctional denervation supersensitivity (to the same extent as seen after cocaine, 10^{-6}M treatment (Johansson & others, 1970). This might reflect a post-

junctional action of LU 3-010 like that previously described for cocaine (Maxwell, Wastila & Eckhardt, 1966).

The membrane pump mechanism is regarded as one important pathway of transmitter removal from the junction gap. Inhibition of this mechanism might therefore increase the "overflow" of transmitter diffusing from a tissue during nerve stimulation. In the present experiments with ^{3}H-NA an increased output of radioactivity would then be expected. However, administration of LU 3-010, 10^{-7} and 3×10^{-7} g/ml, resulted in only a slight increase, if any, in the output of radioactivity at nerve activity in spite of the fact that these drug concentrations apparently give an effective inhibition of the membrane pump. The relative importance of the neuronal uptake mechanism is dependent on the conditions for diffusion (cf. Häggendal, 1970), which are very favourable in the thin portal vein suspended in Krebs solution. Thus, it is probable that under the present experimental conditions the possibilities for diffusion of the transmitter to the superfusate are so good that inhibition of the membrane pump mechanism only plays a minor role for the output of noradrenaline. A further support for this view is the fact that there was no change in the metabolic pattern of the output of radioactivity after LU 3-010. However, a decrease in the transmitter output was observed at 10^{-6} g/ml of LU 3-010 which indicates that the drug in high concentration also interferes with the transmitter release. It is possible that this effect on the transmitter release to some extent might also contribute to the lack of a clear-cut increase in the output of radioactivity at concentrations of LU 3-010, giving effective membrane pump inhibition.

The finding that LU 3-010 can produce an inhibitory effect both on the neuronal uptake and on the transmitter release dependent on the concentration might explain why LU 3-010, 10^{-7} g/ml, slightly potentiated mechanical responses to nerve stimulation whereas concentrations of 10^{-6} g/ml and higher significantly reduced the neurogenic responses (Figs 2 and 3). The favourable diffusion conditions in this preparation make it reasonable that the potentiation of responses to nerve stimulation by selective membrane pump inhibition would be small or lacking. A small potentiation is seen in Fig. 2 after LU 3-010, 10^{-7} g/ml, whereas in Fig. 3 such an effect is not apparent, which might be explained by the different experimental time schedules used.

Since the release of transmitter was decreased at LU 3-010, 10^{-6} g/ml, it is reasonable that the release would be further decreased with higher drug concentrations. Consequently, the pronounced reduction in neurogenic responses observed when the LU 3-010 concentration was increased to 10^{-5} g/ml could be due to failing transmitter output. However, the responses to exogenous noradrenaline were diminished at LU 3-010, 10^{-5} g/ml. Thus, there must also be an action of the drug at the effector level. A blockade of the α-adrenoceptor by LU 3-010 in this high concentration can hardly explain this finding since the spontaneous activity, which is not dependent upon intact adrenergic nerves (Johansson & Ljung, 1967), is markedly affected. Furthermore, the responses to increased potassium concentrations were diminished at this high LU 3-010 concentration. The amplitudes of the spontaneous activity and of the phasic responses to a three-fold increase in the potassium concentration and the contracture elicited by an eight-fold increase were markedly diminished. Since these contractions are independent of adrenergic mechanisms it is evident that LU 3-010, 10^{-5} g/ml, exerts a direct action on the smooth muscle. This agrees with the findings of Hrdina & Ling (1970) that

desipramine interferes with the availability of calcium for the contractile process in vascular smooth muscle. It is possible that the increase in contraction frequency caused by LU 3-010 (Fig. 4) reflects an increased membrane excitability of the smooth muscle. Such an action might potentiate the effect of noradrenaline and offer one possible explanation for the finding that LU 3-010, 10^{-6} g/ml, tended to displace the concentration-effect curve for noradrenaline to the left more than does chronic denervation (cf. Fig. 1 and Johansson & others, 1970). Also, the influence of LU 3-010 on the effector cell may be complex, involving an increased membrane excitability and an inhibitory action at some later step in the excitation-contraction coupling.

It is clear that difficulties might arise when thymoleptic drugs are used for quantitative studies of transmitter release and inactivation in peripheral neuroeffector units. In addition to the desired inhibition of the neuronal uptake mechanism, the drug LU 3-010 also interferes with the transmitter release and with the function of the effector cells, effects which become apparent at different LU 3-010 concentrations. It seems possible that other thymoleptic drugs also exert these effects. Thus it is of great interest that the relation between plasma concentrations of nortriptyline and therapeutic effect in endogenous depression is curvilinear, with less response to both extremely low and extremely high plasma concentrations of the drug (Åsberg, Cronholm & others, 1971). Also the complex and fluctuating symptoms at intoxication with thymoleptic drugs may partly be associated with such a multiple mode of action as discussed for LU 3-010.

Acknowledgements

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