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THE ROLE OF CALCIUM IN THE EFFECTS OF NORADRENALINE AND PHENOXYBENZAMINE ON ADRENERGIC TRANSMITTER RELEASE FROM ATRIA: NO SUPPORT FOR NEGATIVE FEEDBACK OF RELEASE

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1 The relation of calcium ion influx into nerve terminals to presynaptic adrenoceptor function and the possible masking, by desensitization due to intraneuronal calcium accumulation, of the effects of adrenoceptor agonists and antagonists on presynaptic α-adrenoceptors was investigated in guinea-pig atria previously incubated with [^{3}H]-noradrenaline.

2 Atria were stimulated with 100 pulses at various frequencies (1 to 15 Hz) in standard (2.3 mM), low (0.26 mM) and high (6.9 mM) calcium-Krebs solution in the absence and then the presence first of noradrenaline and subsequently phenoxybenzamine.

3 The per pulse overflow of tritium was directly related to the calcium concentration of the Krebs solution, being much reduced and substantially increased in 0.26 and 6.9 mM calcium-Krebs solutions respectively.

4 Noradrenaline inhibited the overflow of tritium in low calcium-Krebs solution, to a relatively constant extent, independently of frequency. In addition, the agonist had a greater maximal inhibitory effect in standard than in reduced calcium-Krebs. The catecholamine was as effective an inhibitor of overflow at the lowest and highest frequencies in high as it was in standard calcium-Krebs solution. Phenoxybenzamine invariably increased the tritium overflow but was generally less effective both in low and in high calcium-Krebs solution. The patterns of inhibition and enhancement of stimulation-induced tritium overflow by these two agents do not indicate an intimate relationship between calcium influx and adrenoceptor activation; nor does desensitization appear to be an adequate explanation of the relationship between frequency of stimulation and the intensity of agonist and antagonist effect in the three different calcium concentrations.

5 It is concluded that the perineuronal levels of adrenergic transmitter do not establish the magnitudes of effect of exogenous adrenoceptor agonists and antagonists on tritium overflow and that a negative feedback regulation of release by transmitter is exceedingly unlikely under ordinary conditions of neurotransmission.

Introduction

The opposing effects of noradrenaline and phenoxybenzamine on the quantity of ^{3}H-transmitter released from sympathetic nerves upon electrical stimulation, determined as tritium overflow, has been explained by invoking the hypothesis that these agents interact as agonist and antagonist with a perineuronal negative feedback system modulating transmitter release. However, several recent reports have emphasized that neither of these agents satisfies the requirements of presynaptic receptor theory (Kalsner, 1979a, b; 1980a; Chan & Kalsner, 1979a,b; Kalsner & Chan, 1979; 1980; Kalsner, Suleiman & Dobson, 1980). A possible explanation for certain of the discrepancies between theoretical prediction and experimental observation is that an accumulation of calcium at critical intraneuronal sites leads to a desensitization and consequent disruption of the autoinhibitory system, rendering it non-functional under specific experimental conditions.

Such an explanation seems unlikely under the modest parameters of stimulation employed in work in my laboratory and additionally raises concern as to the physiological relevance of any supposed regulatory system which fatigues so readily under mild conditions of excitation. However, since the direct participation of calcium in presynaptic regulation is an unsettled issue it seemed fruitful to investigate the effects of low and high external calcium concentra-

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tions on the patterns of drug-induced inhibition and enhancement of tritium overflow. Further, the data were assessed to determine if they are interpretable as support for a model of transmitter release incorporating a process of negative feedback.

Methods

Tissue preparation

Immediately after death by cervical dislocation, hearts were removed from albino guinea-pigs (300–600 g) of either sex and immersed in cold (4°C) and oxygenated (95% O_2:5% CO_2) Krebs-Henseleit (Krebs) solution (NaCl 115.3, KCl 4.6, $CaCl_2$ 2.3, $MgSO_4$ 1.1, $NaHCO_3$ 22.1, KH_2PO_4 1.1, glucose 7.8 and disodium edetate 0.03 mM). Left atria were removed, bisected from base to apex as previously described (Furchgott & Garcia, 1968; Furchgott, Garcia, Wakade & Cervoni, 1971; Kalsner *et al.*, 1980) and incubated for 60 min in 4.0 ml of oxygenated Krebs solution containing (−)-[7,8-^{3}H]-noradrenaline (10 μCi/ml, 7.6–10.0 × 10^{-7}M) at 37°C. Following incubation the tissues were briefly washed with fresh Krebs solution and then mounted under 2 g tension between platinum electrodes in a superfusion apparatus. The preparations were continously superfused with warmed (37°C) and oxygenated Krebs solution by a gravity feed system which maintained a constant flow rate of 5 ml/min.

Stimulation parameters and protocols

Following a 90 min equilibration period, atria in standard calcium-Krebs solution were stimulated transmurally via Grass model S5 stimulators at 9 min intervals with trains of 100 biphasic pulses, 1.0 ms duration and at supramaximal voltage, once at each of the test frequencies (1, 2, 5 and 15 Hz) in random sequence. The identical test sequence was repeated a second (S_2) and third (S_3) time in tissues serving as controls for the experimental protocols described below.

Where the effects of low (0.26 mM) or high (6.90 mM) calcium ion concentrations were examined, the appropriately modified Krebs solution was superfused onto one of each pair of atrial halves 30 min before the first stimulation period (S_1) and maintained throughout S_2 and S_3. Noradrenaline (0.3 μg/ml; 1.8 × 10^{-6} M) where used, was incorporated directly into the Krebs solution having the appropriate calcium concentration, and tissues were exposed to the agonist for 20 min before and throughout the second series of stimulations (S_2) which were identical to the first. After a 20 min washout period with agonist-free Krebs, atrial halves were exposed for 30 min to phenoxybenzamine (10 μM) previously dissolved in standard calcium (2.3 mM)-Krebs solution. This was followed by a 20 min washout period with Krebs having the test calcium concentration used in S_1 and S_2 and the third series of stimulations (S_3) was performed. Matching control atria, not exposed to phenoxybenzamine, were also returned to standard calcium conditions, for a 30 min period. Cocaine (8.8 × 10^{-6} M) and normetanephrine (1 × 10^{-5} M) were always present in the Krebs solutions to eliminate any complications arising from neuronal or extraneuronal uptake of noradrenaline.

Drugs and radiochemicals

The drugs used and their sources were: cocaine hydrochloride (Allen & Hanburys), (±)-normetanephrine hydrochloride (Calbiochem), (−)-noradrenaline bitartrate (Calbiochem), and phenoxybenzamine hydrochloride (Smith, Kline & French). The radioisotope (−)-[7,8-^{3}H]-noradrenaline hydrochloride (specific activity 10–13 Ci/mmol) was obtained from the Radiochemical Centre, Amersham. It was diluted to a stock concentration of 100 μCi/ml in (−)-ascorbic acid (50 μg/ml) and stored in 5 ml aliquots at 4°C under nitrogen gas. To obtain a final concentration of 10 μCi/ml (7.6–10.0 × 10^{-7} M) in the incubation medium, 0.4 ml of this stock was added to 3.6 ml of Krebs solution.

Overflow of tritium

The overflow of tritium from the preparations was determined by counting 1.0 ml aliquots of the 15.0 ml superfusate collected in vials by a fraction collector which rotated once every 3 min. The aliquots were then transferred to vials containing 10 ml of Aqueous Counting Scintillant (Amersham) and counted to a 1% error in a Beckman LS-230 counter with automatic external standardization to determine efficiency.

Basal overflow is expressed as disintegrations per min (d/min) and referred to as the total radioactivity detected in the 3 min sample collected immediately before each stimulation. Stimulation-induced overflow was calculated as the difference between basal overflow and the total d/min detected in the 3 min sample collected during and after stimulation. Transmural stimulation was always begun at the start of a 3 min collection period.

Mean data on overflow are presented with their standard errors and Student's t test was used for all comparisons between treated and untreated atrial halves. P values of less than 0.05 were considered significant. To assess the relationships between overflow of tritium and frequency and the effects of agents at the three concentrations of calcium employed in this study, the correlation coefficient (r) was calculated as described by Schefler (1979). For example, the correlation between overflow per pulse and fre-

quency was found to be highly significant ($P < 0.01$), and evaluation of r^2 indicated that from 85% to 93% of the change in overflow per pulse is accounted for by increasing frequency.

Results

Stimulation-induced overflow

The absolute overflow of tritium per stimulation pulse in superfused guinea-pig atria is clearly calcium-dependent (Figure 1). In atria exposed to the low calcium (0.26 mM) medium the overflows of radioactivity were very substantially reduced, at all test frequencies, below that obtained in standard Krebs (2.3 mM calcium) and they were materially elevated in the high calcium medium (6.9 mM) (Figure 1). For example, the per pulse overflow at 1 Hz was doubled in high calcium and reduced by about 85% in low calcium-Krebs solution. These approximate overflow relationships held over much of the frequency range.

The per pulse overflow of tritium increased as the frequency of stimulation with 100 pulses increased from 1 to 15 Hz in the standard 2.3 mM calcium-Krebs solution ($r = 0.966$, $P < 0.01$). A facilitation of overflow with frequency was also observed in the low calcium ($r = 0.923$, $P < 0.01$) and in the high calcium Krebs media ($r = 0.964$, $P < 0.01$).

Effect of noradrenaline on overflow

In atria stimulated at 1, 2, 5 and 15 Hz with 100 pulses in the presence of noradrenaline (0.3 μg/ml; 1.8×10^{-6} M), the overflow of tritium in 2.3 mM calcium was significantly diminished compared to values obtained in the same preparations before exposure to the catecholamine (Table 1). The effect of the agonist was statistically assessed by comparison of the overflow ratios between first and second stimulation runs at each frequency in control and agonist-treated atrial halves. The inhibitory effect of the agonist was most pronounced at the lowest frequency and declined progressively as the frequency of stimulation rose ($r = 0.994$, $P < 0.001$).

To compare the efficacy of noradrenaline and

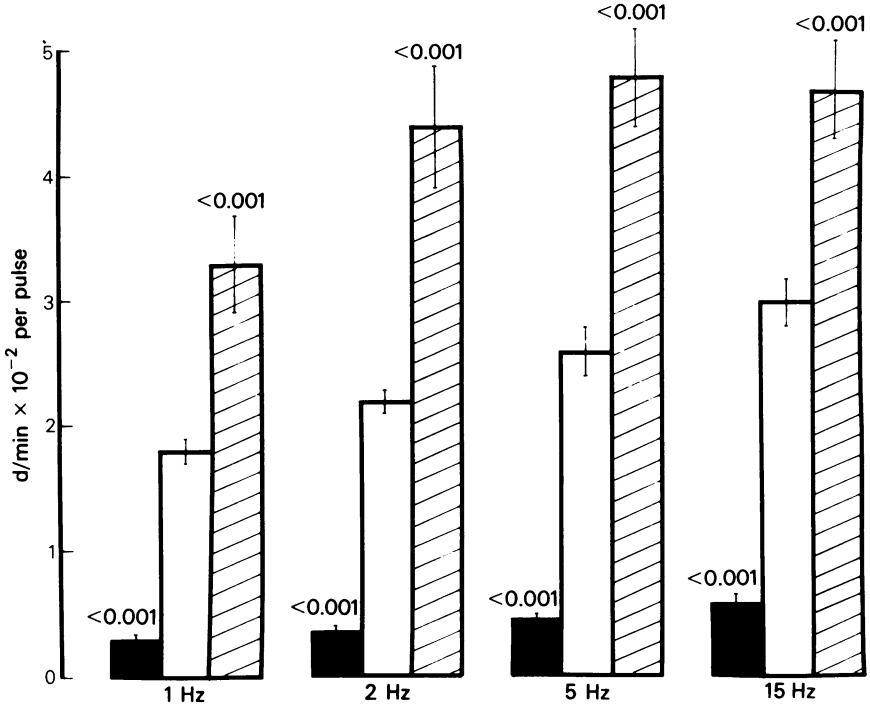

Figure 1 Relationship between stimulus frequency and the overflow of tritium per pulse in Krebs solution of three different calcium concentrations. Stimulation was with 100 pulses at each test frequency. Solid column indicates 0.26 mM, open column 2.3 mM and hatched column 6.9 mM calcium, present in the bathing medium. The number of values at each calcium level was 17, 41 and 24, respectively. Vertical lines show s.e.mean. *P* values shown above columns refer to comparisons with values shown for corresponding group in 2.3 mM calcium-Krebs solution.

Table 1 Ratios of transmitter overflow in the second period of stimulation (S_2) and the first period (S_1) in the absence and presence of noradrenaline (NA)

Calcium concentration	*Experimental group*	*No. of values*	*Overflow ratio: S_2/S_1*			
(mM)	(a,b)		1 Hz	2 Hz	5 Hz	15 Hz
0.26	Control	8	0.79 ± 0.10	0.82 ± 0.09	0.94 ± 0.12	1.03 ± 0.15
	NA	9	0.54 ± 0.10*	0.66 ± 0.13	0.53 ± 0.09	0.52 ± 0.08*
			(0.42 ± 0.08)	(0.54 ± 0.11)	(0.49 ± 0.08)	(0.54 ± 0.08)
2.30	Control	20	1.06 ± 0.06	1.08 ± 0.05	0.98 ± 0.03	0.97 ± 0.05
	NA	21	0.36 ± 0.03	0.52 ± 0.03	0.67 ± 0.04	0.72 ± 0.04
			(0.39 ± 0.04)	(0.56 ± 0.03)	(0.66 ± 0.04)	(0.70 ± 0.04)
6.90	Control	12	0.96 ± 0.08	0.84 ± 0.05	0.85 ± 0.07	0.91 ± 0.07
	NA	12	0.43 ± 0.04	0.62 ± 0.05*	0.87 ± 0.04**‡	0.78 ± 0.05‡
			(0.41 ± 0.04)	(0.52 ± 0.05)	(0.74 ± 0.04)	(0.71 ± 0.05)

[a]Noradrenaline, when given, was administered in the interval between S_1 and S_2 as described in text. [b]Mean overflow ratios shown in parentheses for noradrenaline-treated groups are those uncorrected for spontaneous changes in efflux (deviation from 1.0) observed in matching control preparations without noradrenaline, as shown. Correction was done by dividing each individually obtained ratio in the given experimental group by the appropriate corresponding mean control group ratio (S_2/S_1). * Indicates value significantly different from that of similarly treated group in standard calcium-Krebs with a $P < 0.05$. ** Indicates value significantly different from that of similarly treated group in standard calcium-Krebs with a $P < 0.01$. ‡ Indicates value significantly different from that of similarly treated group in low (0.26 mM) calcium-Krebs.

Table 2 Ratios of transmitter overflow of atria described in Table 1 in the third period of stimulation (S_3) and the first period (S_1) in the absence and presence of phenoxybenzamine (Pbz)

Calcium concentration	*Experimental group*	*No. of values*	*Overflow ratio: S_3/S_1*			
(mM)	(a,b)		1 Hz	2 Hz	5 Hz	15 Hz
0.26	Control	8	1.00 ± 0.14	0.98 ± 0.14	0.98 ± 0.09	1.23 ± 0.14
	Pbz	9	1.50 ± 0.22*	1.70 ± 0.31*	1.86 ± 0.27	1.55 ± 0.22
			(1.50 ± 0.22)	(1.67 ± 0.31)	(1.82 ± 0.26)	(1.91 ± 0.27)
2.30	Control	20	1.01 ± 0.07	1.02 ± 0.06	1.02 ± 0.06	1.04 ± 0.04
	Pbz	21	3.06 ± 0.27	3.04 ± 0.24	2.32 ± 0.16	1.66 ± 0.11
			(3.09 ± 0.27)	(3.10 ± 0.24)	(2.37 ± 0.16)	(1.73 ± 0.11)
6.90	Control	12	0.86 ± 0.09	0.80 ± 0.05	0.80 ± 0.05	0.69 ± 0.04
	Pbz	12	2.34 ± 0.30†‡	2.50 ± 0.29	1.92 ± 0.23	1.71 ± 0.18
			(2.02 ± 0.26)	(2.00 ± 0.23)	(1.53 ± 0.18)	(1.18 ± 0.12)

[a] Phenoxybenzamine, when given, was administered in the interval between S_2 and S_3 as described in text. [b]Mean overflow ratios shown in parentheses for phenoxybenzamine-treated groups are those uncorrected for spontaneous changes in efflux (deviation from 1.0) observed in matching control preparations without phenoxybenzamine, as shown. Correction was done by dividing each individually obtained ratio in the given experimental group by the appropriate corresponding mean control group ratio (S_3/S_1). * Indicates value significantly different from that of similarly treated group in standard calcium-Krebs with a $P < 0.01$. † Indicates value not quite ($0.1 > P > 0.05$) significantly different from that of similarly treated group in standard calcium-Krebs. ‡ Indicates value significantly different from that of similarly treated group in low (0.26 mM) calcium-Krebs.

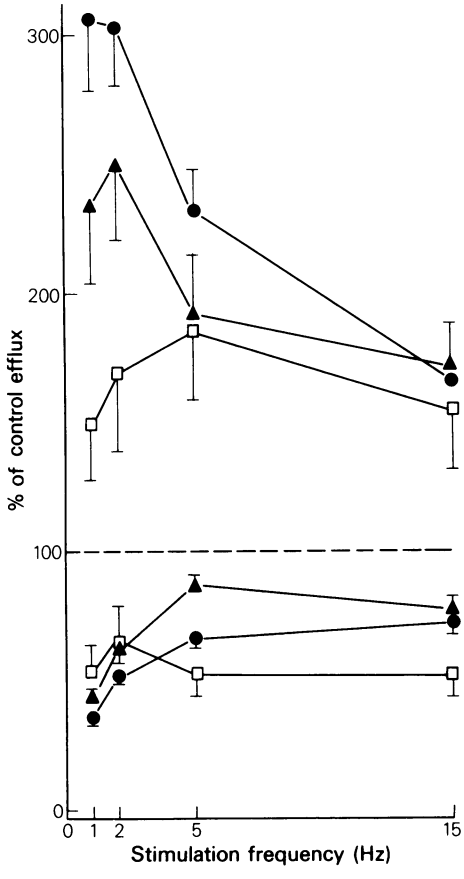

Figure 2 The effects of noradrenaline and phenoxybenzamine on the stimulation-induced overflow of tritium in guinea-pig atria in 0.26 mM (□), 2.3 mM (●) and 6.9 mM (▲) calcium-Krebs solution. Inhibition by the agonist (1.8×10^{-6} M) appears as values below the 100% control line and enhancement by the antagonist (10 μM for 30 min) as values above the 100% control line. Vertical lines show s.e.mean. Number of values in each group, statistical comparisons and methodology are described in the legends to Tables 1 and 2.

phenoxybenzamine at different calcium concentrations the overflow ratios in treated atria were corrected for any time-dependent spontaneous changes in overflow occurring in untreated atrial halves during the course of the experiment (S_1, S_2 and S_3) in high, low or standard calcium-Krebs solutions (Tables 1 and 2). In the low calcium-Krebs, noradrenaline inhibited the overflow significantly less at the lowest but more at the highest test frequency as compared to values obtained in standard Krebs solution (Figure 2). The peak inhibitory effect in low calcium however, was substantially less than that obtained in 2.3 mM calcium-Krebs solution. Additionally, the effect of noradrenaline did not decrease with frequency in 0.26 mM calcium as it did in 2.3 mM calcium but remained relatively constant over the entire frequency-overflow range ($r = 0.206$, $P > 0.1$). The effect of noradrenaline was reduced significantly in high calcium-Krebs only at the two intermediate test frequencies (Table 1 and Figure 2).

Effect of phenoxybenzamine on overflow

In 2.3 mM calcium-Krebs solution the maximal increase of stimulation-induced overflow by phenoxybenzamine treatment, as described in Methods, occurred at 1–2 Hz and was less at the higher test frequencies, as determined by comparison of overflow ratios in the absence (S_1) and presence (S_3) of the haloalkylamine (Table 2 and Figure 2). In 0.26 mM calcium-Krebs solution, the enhancing effect of phenoxybenzamine on stimulation-induced tritium overflow appeared smaller than in standard calcium at all test frequencies but the differences were not quite statistically significant at 5 and 15 Hz. The overflow ratios (S_2/S_1) at 1, 2, 5 and 15 Hz, in the presence of phenoxybenzamine, did not differ significantly from each other. The maximal enhancement of overflow by the antagonist in the low calcium medium was less than 1 fold in contrast to a 2.1 fold increase in the standard calcium medium (Table 2). In 6.9 mM calcium-Krebs solution, the effect of phenoxybenzamine on stimulation-induced overflow, after correction for the spontaneous decline in overflow with time in control atria maintained in high calcium, was not significantly diminished from values obtained in standard calcium-Krebs solution (Table 2 and Figure 2). Although the effect of the α-adrenoceptor antagonist declined significantly with frequency in 2.3 mM ($r = 0.845$, $P < 0.05$) and almost significantly in 6.9 mM calcium ($r = 0.763$, $0.1 > P > 0.05$), no such frequency-dependent effect was seen in 0.26 mM calcium ($r = 0.469$, $P > 0.1$).

Spontaneous overflow of tritium

The basal overflow of tritium, measured immediately before each stimulation did not differ significantly in 2.3 and 0.26 mM calcium but was substantially raised in 6.9 mM calcium, the values being 10.0 ± 0.2, 9.7 ± 0.4 and 54.2 ± 7.3 d/min $\times 10^{-3}$, respectively, during the initial (S_1) phase of the experiment; by the termination of the experiment they had declined to 6.5 ± 0.2, 6.7 ± 0.3 and 25.6 ± 1.4 d/min $\times 10^{-3}$.

Discussion

The present findings clarify somewhat the involvement of calcium in the modulating effects of noradrenaline and phenoxybenzamine on adrenergic

transmitter release. The role of the ion appears to be less direct than was previously thought (e.g. see review by Starke, 1977). In addition, information is provided here on the purported relationship between a supposed intraneuronal calcium accumulation, presynaptic receptor desensitization and the frequency-dependent character of the effects of adrenoceptor agonists and antagonists on stimulation-induced transmitter overflow.

The intraneuronal flux of extracellular calcium ions is an essential element in neuronal secretion of transmitter although its precise relationship to the release process is unclear (Rahamimoff, 1970; Kirpekar, 1975; Blaustein, Ratzlaff & Kendrick, 1978). Additionally, each nerve impulse is presumed to leave some calcium bound to an active internal site (Younkin, 1974; Starke, 1977) and the briefer the interval between stimulation pulses the greater the residual calcium at the onset of the next pulse, supposedly accounting for the sometimes observed facilitation of output with increasing frequency (e.g. Hughes, 1972; Chan & Kalsner, 1979a). Kirpekar (1975) suggested that intraneuronal accumulation of calcium may also account for the refractoriness of release during intensive nerve stimulation.

It has been proposed that 'activation of presynaptic alpha adrenoceptors leads to a decrease; and their blockade to an increase, in the availability of calcium for stimulation-secretion coupling' and that during high rates of impulse flow, changes in the intraneuronal calcium level due to presynaptic receptor activation occur within the 'saturation range' (Starke, 1977). Under the latter conditions, modulators of calcium influx would have minimal effects during stimulation. The decreased presynaptic efficacy of noradrenaline and phenoxybenzamine, at high frequencies of stimulation, is thus thought by some to be attributable to desensitization of a calcium-coupled α-adrenoceptor-mediated inhibitory system (Langer, 1977; Starke, 1977; Westfall, 1977). An assessment of the extended supposition (e.g. Langer, 1977) that receptor desensitization explains the declining efficacy of agents which interact with presynaptic α-sites, even when only a moderate frequency range and a modest pulse number are employed, is imperative since it has serious implications for the applicability of presynaptic receptor theory to routine transmitter release processes *in vivo* and to the amenability of the theory to experimental scrutiny.

The experimental support for a direct link between presynaptic α-receptor activation and inhibition of calcium influx is limited to very few concrete reports (Langer, Dubocovich & Celuch, 1975; Marshall, Nasmyth & Shepperson, 1977; Drew, 1978). In particular, Langer *et al.* (1975) found, using cat spleen, that reduction of the extracellular calcium to 0.26 mM raised the efficacy of both noradrenaline and phenoxybenzamine as, respectively, inhibitor and promoter of transmitter efflux at 5 and 30 Hz, frequencies at which they were ineffective, or poorly effective, in standard Krebs solution.

The strict dependence of the per pulse overflow of transmitter on the absolute level of extracellular calcium, as seen in the present experiments, demonstrated that the manipulations of the external calcium level were successful and it serves as a probable model from which to anticipate the behaviour of other systems which might also be calcium-dependent. The overflow of tritium was greater the higher the extracellular calcium level at each of the four experimental test frequencies but no such definitive relationship, inverse or otherwise, was detected between calcium concentration and the effectiveness of presynaptic modulators of release. Furthermore, previous studies did not consider the qualitative effects of calcium on the per pulse overflow of transmitter and the consequences of this for an accurate assessment of the linkage between extracellular calcium and presynaptic receptor mechanisms.

The present experiments done in standard calcium (2.3 mM) Krebs solution confirm previous findings that noradrenaline inhibits the stimulation-induced overflow of tritium less at higher than at lower frequencies (Kalsner, 1979b; 1980a; Chan & Kalsner, 1979b; Kalsner & Chan, 1979; Kalsner *et al.*, 1980). If noradrenaline indeed acts to modulate rates of calcium translocation (Starke, 1977), two critical observations are predicted when the extracellular calcium is reduced: (a) the extent of the overflow inhibition induced by a given amount of noradrenaline would be increased at low frequencies since ion channels would be more effectively closed against the reduced calcium influx during stimulation; and at high frequencies the agonist would be more effective, both for this reason and because desensitization attributable to high rates of calcium translocation (or to an excessive intraneuronal accumulation of calcium) should be attenuated or eliminated: and (b) the frequency-dependent character of the effect of noradrenaline on the overflow pattern, undistorted by desensitization, would express much more accurately the competition for occupancy of a finite population of presynaptic α-receptor sites between exogenous noradrenaline and the progressively increasing levels of neurally released amine.

Noradrenaline had a greater effect in reduced calcium-Krebs solution only at the highest test frequency. When the range of frequencies is examined, the data do not sustain an intimate coupling of calcium to the presynaptic action of noradrenaline (Table 1). For example, the maximum percentage inhibition by noradrenaline in 2.3 mM calcium-Krebs was greater than that in 0.26 mM calcium-Krebs solution, not in keeping with the suggestion that desensitization is a pre-eminent distorting factor in standard calcium-Krebs, nor with a mechanism of agonist

action tightly linked to inhibition of calcium translocation.

Since calcium ion is essential to transmitter release processes a serious reduction in extracellular calcium was expected to depress profoundly the amount of transmitter released per pulse and this was observed (measured as tritium overflow) at all frequencies in 0.26 mM calcium-Krebs solution. Consequently, the number of presynaptic receptor sites occupied by endogenously released transmitter, at any given frequency, is reduced and the exogenously administered agonist should therefore have a greater effect in the low calcium solution as more receptors are available for it to occupy and activate. This reinforces the enhanced inhibitory action expected from a more efficient closing of calcium channels and a diminished desensitization process. Such a pattern of increased efficacy, it should be reiterated, was not observed.

If the pattern of effectiveness of noradrenaline in low calcium-Krebs solution, undistorted by desensitization, represents the competition between endogenous and exogenous catecholamine for presynaptic α-adrenoceptor sites, a challenge to the hypothesis of negative feedback becomes apparent. As the frequency of stimulation climbs towards 15 Hz with a fixed pulse number (100) the total transmitter release process becomes increasingly compressed in time, from a maximum of 100 to a minimum of 6.7 s, resulting in a higher mean perineuronal concentration of active transmitter during stimulation. This was shown previously to be the case by an analysis of the magnitude of effector responses with rising frequency (Chan & Kalsner, 1979a). For this reason, a fixed amount of exogenous noradrenaline must become a decreasing proportion of the total noradrenaline incident on the presynaptic receptors during the stimulation period as the frequency rises. The effectiveness of the added noradrenaline, measured as a decrease in the stimulation-induced overflow of tritium, should therefore decline with frequency. However, no inverse relationship between frequency and agonist efficacy occurred in reduced calcium-Krebs solution when overflow ratios are compared at each given frequency in the presence and absence of added noradrenaline. This is so even though per pulse overflow itself also rises with frequency, which should magnify the quantitative discrepancy between free and active transmitter levels at low and high frequencies. As shown in Figure 2 and Table 1, a relatively constant degree of overflow inhibition was seen with noradrenaline in 0.26 mM calcium-Krebs solution, regardless of stimulation frequency, indicating that the amount of transmitter released per unit time (assumed to be proportional to the measured overflow) is not the critical determinant of presynaptic adrenoceptor function.

The findings made with phenoxybenzamine in 0.26 mM calcium-Krebs are germane to an evaluation of presynaptic receptor theory. An enhancement of tritium overflow by this drug is what would be predicted by this hypothesis for an antagonist of noradrenaline. Moreover, exogenous noradrenaline should have less effect and phenoxybenzamine more effect on the absolute efflux as the frequency increases, because of the increasing perineuronal level of endogenous transmitter. However, phenoxybenzamine was less effective at the higher frequencies in standard calcium-Krebs; if desensitization contributed to the lower efficacy it ought to have been minimal in low calcium-Krebs solution. Additionally, the more efficient closing of calcium channels by transmitter noradrenaline at low frequencies in 0.26 mM calcium-Krebs should also help to reveal a greater effect with phenoxybenzamine. Complications arise in the interpretation of these data because of the decreased per pulse overflow of tritium in a low calcium solution. Not withstanding the fact that the relative magnitudes of these projected variables cannot be ascertained at each frequency, the finding of a generally diminished rather than an enhanced effect of phenoxybenzamine in reduced calcium-Krebs, even at the highest frequencies, argues against the suggestion that desensitization is a significant factor in blunting the magnitude of the phenoxybenzamine effect in standard calcium-Krebs solution. In fact, the peak enhancement by the haloalkylamine is reduced from 206% (in standard calcium) to 86% (in low calcium) above the corresponding control values.

The crucial finding which militates against the hypothesis is the lack of relationship in low calcium-Krebs between the frequency of stimulation and the size of the increased overflow caused by phenoxybenzamine. It would be anticipated that the effect should have been greatest at 15 Hz and least at 1 Hz, but instead the effect of the antagonist, like that of the agonist, was generally independent of frequency; the only variations between frequencies were statistically non-significant.

By contrast, in 6.9 mM calcium-Krebs solution, an accelerated desensitization process together with calcium channels, supposedly less effectively closed by noradrenaline, should contribute to a reduced general efficacy of the antagonist. This, in turn, would be reinforced by the altered proportions of exogenous and neurally liberated amine, because of the elevated per pulse overflow of transmitter. In fact, the activity of phenoxybenzamine declined less steeply in high than it did in standard calcium-Krebs and although a general trend to decreased effectiveness was detected in high calcium-Krebs , it was more pronounced at the low than at the high frequencies, the converse of what would be anticipated based on the per pulse overflow-frequency relationships and an accelerated desensitization. However, what is of special interest is that the effectiveness of noradrenaline at the two lowest frequencies (1 and 2 Hz) did not

differ in the two extremes of calcium (0.26 mM and 6.9 mM) and at the highest test frequency of 15 Hz in 6.9 mM calcium-Krebs, where desensitization would be most acute, its effect is not diminished compared to values obtained in 2.3 mM calcium. These observations reduce the likelihood that desensitization and an α-adrenoceptor-mediated process, both linked tightly to calcium kinetics are predominant parameters in establishing the overflow pattern in the presence of noradrenaline. Additionally, the finding that noradrenaline was no less effective an inhibitor of overflow at the lowest or at the highest frequency in 6.9 mM calcium than it was in standard calcium-Krebs is incompatible with the hypothesis of competition between locally released and added noradrenaline for a common pool of receptors.

The present study aimed to assess the contribution of desensitization to the patterns of activity of noradrenaline and phenoxybenzamine on tritium overflow. It is apparent that desensitization cannot be an adequate explanation for the failure of each drug to show the type of activity predicted from the negative feedback hypothesis. Apart from this failure, it is worth noting that if desensitization is the decisive outcome of activating a negative feedback system under conditions of modest frequency and pulse number then the system is rendered inoperative when it is most required. A negative feedback system supposedly serves to dampen moderate and high levels of activity, not shut-down exclusively the lowest levels. It was shown previously (Kalsner, 1979b) that even with only 4 stimulation pulses, given at 1, 5 and 15 Hz, no association was noted between the perinueronal transmitter level (as assessed by effector responses) and the size of the increase in tritium overflow induced by phenoxybenzamine. Interestingly, because at all three calcium levels there was a direct relationship between tritium overflow per pulse and stimulation frequency, there appears to be no dependence of these increases on residual calcium at an intraneuronal locus.

Other recent work from my laboratory has challenged the negative-feedback function of presynaptic sites (Kalsner, 1979a, b; 1980a; Chan & Kalsner, 1979a, b; Kalsner *et al.*, 1980) and even their characterization as α- and β-adrenoceptors (Kalsner & Chan, 1979; 1980; Kalsner, 1980b). A premature assignment of specificity and function may have been made with regard to presynaptic loci of drug interaction; one that has been more conservatively withheld from numerous postsynaptic sites of dubious function located on and in a variety of autonomic effectors.

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