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THE RELEASE OF NORADRENALINE FROM SYMPATHETIC FIBRES IN RELATION TO CALCIUM CONCENTRATION

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In a previous paper (Burn & Gibbons, 1964) we described evidence suggesting that the release of noradrenaline from sympathetic fibres was dependent on the concentration of Ca^{2+} outside the fibre. This dependence of the amount of noradrenaline released on Ca^{2+} resembled the similar dependence of the amount of catechol amines released from the adrenal medulla by acetylcholine (ACh) (Douglas & Rubin, 1961, 1963).

We now describe experiments in which we have investigated the effect of Ca^{2+} on the release of noradrenaline by ACh, nicotine and tyramine in isolated rabbit atria, and we have investigated the effect of Ca^{2+} on the response to noradrenaline in atria. In the previous paper we made observations on the effect of bathing the isolated ileum in Locke's solution which was Ca^{2+}-free for short periods of 2 min, and for longer periods of 20 min. We found that when Ca^{2+} was replaced after a short period, the pendular rhythm returned at once, but when it was replaced after a longer period, the pendular rhythm returned very slowly. We have now made similar observations on strips of ileum taken from rabbits treated with reserpine.

METHODS

We have used the isolated atria of the rabbit heart set up in an organ bath of 100 ml. capacity, filled with Locke's solution. This contained (g/l.) NaCl 8·5, KCl 0·42, $CaCl_2$ 0·24, $NaHCO_3$ 0·5, and dextrose 2·0. The bath was bubbled with 5% carbon dioxide in oxygen and maintained at 29° C.

We also used the isolated ileum preparation of the rabbit described by Finkleman (1930), stimulating the periarterial nerves to produce inhibition of the pendular movements. Rectangular wave supramaximal pulses of 1 msec duration were given. The ileum was suspended in Locke's solution which was bubbled with 5% carbon dioxide in oxygen at 33° C.

In treating rabbits with reserpine, the U.S.P. solution was used and two doses were given by intraperitoneal injection at an interval of 7 hr, each dose being 1·25 mg/kg. The rabbits were killed on the following day. Solutions of acetylcholine bromide, hyoscine hydrobromide, tyramine hydrochloride, nicotine acid tartrate and noradrenaline bi-tartrate were prepared; doses of the first three were expressed in terms of the salts, and of the last two in terms of the base.

RESULTS

Effect of Ca^{2+} on stimulation. An illustration of the effect of raising the concentration of Ca^{2+} on the response to stimulation of post-ganglionic fibres is given in Fig. 1. In this observation, stimulation was applied at the low frequency of 3/sec, giving 200 shocks, and the inhibition produced in the presence of 2mM-Ca^{2+} was small (Fig. 1*a*). At the arrow the Ca^{2+} concentration was raised to 6·6 mM. This quickened the rate of the pendular movements and reduced their amplitude. The same stimulation then produced a much greater inhibition (Fig. 1*c*).

Effect of Ca^{2+} on response to ACh. In the ileum it was difficult to produce an effect like that of stimulation of the post-ganglionic fibres by adding

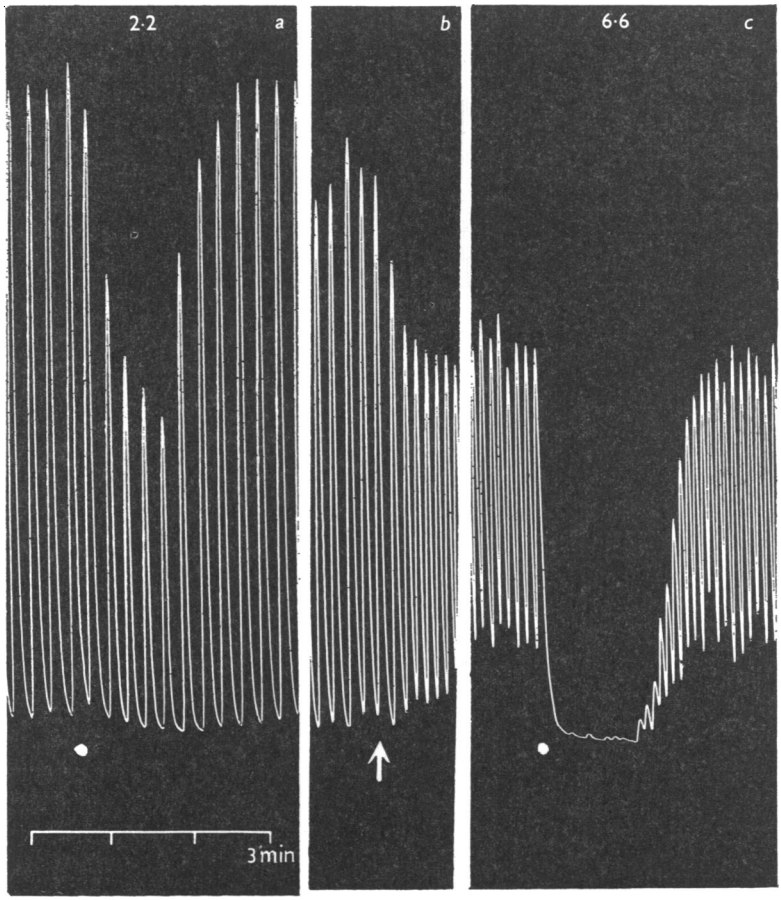

Fig. 1. Isolated rabbit ileum in Locke's solution. In *a* calcium 2·2 mM. At dot stimulation of periarterial nerves by 200 supramaximal shocks at 3/sec. Note small inhibition. In *b* calcium raised to 6·6 mM. In *c* stimulation repeated at dot. Note large inhibition.

ACh to the bath. Even in the presence of hyoscine 10^{-6}, the inhibitory effect of ACh was very brief and was rapidly overcome by a strong contraction. In the atria, however, in the presence of hyoscine, the effect of ACh in slowing the heart was almost abolished. Figure 2 shows the effect of ACh on the rate and amplitude of the isolated atria in the presence of hyoscine 10^{-6} when the Ca^{2+} concentration was 2·2 mM, and then when it was 6·6 mM. In this experiment raising the Ca^{2+} concentration

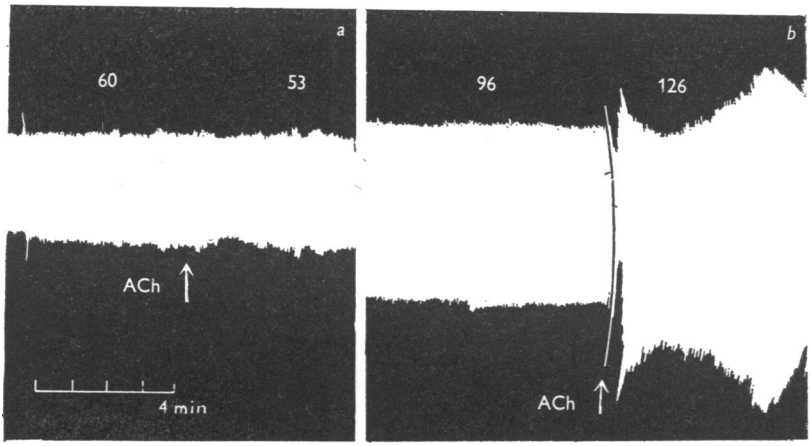

Fig. 2. Isolated rabbit atria, in bath containing hyoscine 10^{-6}. In *a* calcium was 2·2 mM. Atrial rate 60/min, reduced by ACh 50 μg/ml. to 53/min. In *b* calcium was 6·6 mM. Atrial rate 96/min, increased to 136/min by ACh 50 μg/ml.

TABLE 1. Effect of Ca^{2+} concentration on the increase in rate of isolated atria caused by ACh, 5×10^{-5}, in the presence of hyoscine, 10^{-6}

	Percentage change in rate Ca^{2+} concentration (mM)		
Expt. no.	2·2	6·6	13·2
1	−11	+41	.
2	−3	+17	.
3	+4	+1	+90
4	+3	+9	+10
5	−6	+27	+40
Mean	−3	+19	+46

increased the spontaneous rate from 60/min to 96/min, though it did not do so in others. Whereas ACh 5×10^{-5} caused some fall in rate at the lower Ca^{2+} concentration, it increased the rate and amplitude at the higher Ca^{2+} concentration. Since the atrial beat was recorded by a spring lever, the change in amplitude was affected by the natural frequency of the spring, and the record did not accurately represent the change in force of contraction. Other observations with ACh are given in Table 1.

In Expt. 3 the spontaneous rate did not change when the Ca^{2+} concentration was raised, but remained at 70/min at 2·2, 6·6 and 13·2 mM. The addition of ACh did not affect the rate at the two lower concentrations of Ca^{2+}, but increased it at 13·2 mM to 133/min in one trial, and to 144/min in a second trial. In Expts. 1, 2, 3 and 5 ACh caused a much greater increase in rate at both higher Ca^{2+} concentrations, while in Expt. 4 the percentage increase was only slightly greater. The mean result was that ACh diminished the rate by 3 % at 2·2 mM Ca^{2+}, it increased it by 19 % at 6·6 mM and increased it by 46 % at 13·2 mM.

Action of nicotine. In the rabbit ileum, nicotine acting in the presence of hyoscine caused inhibition (Burn & Gibbons, 1964) and this inhibition became greater as the Ca^{2+} concentration rose. A similar observation was made in the atria, where nicotine acted like ACh, though in smaller dose. A concentration of nicotine 2×10^{-6} increased the atrial rate from 68 to 92 per min when the Ca^{2+} concentration was 2·2 mM, but increased it from 67 to 136 when the concentration was 4·4 mM.

TABLE 2. Effect of Ca^{2+} concentration on the increase in rate of isolated atria caused by tyramine, in concentration 2×10^{-6}

	Percentage change in rate Ca^{2+} concentration (mM)	
Expt. no.	2·2	6·6
6	+48	+58
7	+49	+62
8	+37	+34
Mean	+45	+51

Action of tyramine. The increase of atrial rate caused by tyramine was almost unaffected by a rise in the Ca^{2+} concentration. Results of three experiments are given in Table 2. In Expts. 6 and 7 tyramine had a slightly greater effect in the higher Ca^{2+} concentration, and in Expt. 8 a slightly smaller effect. The mean percentage increase caused by tyramine was 45 for 2·2 mM Ca^{2+}, and was 51 for 6·6 mM-Ca^{2+}. The difference was small, compared with the difference for ACh, which in a Ca^{2+} concentration of 2·2 mM usually caused no rise at all.

The effect of Ca^{2+} on noradrenaline. The response of the atrial rate to noradrenaline itself was examined to see if it was modified by a rise in Ca^{2+} concentration. The results were very variable, and observations made in five preparations are recorded in Table 3. In four observations it appeared that the increase in rate was greater in 6·6 mM-Ca than in 2·2 mM, but when the mean result was determined, there was no appreciable difference.

The effect of Ca^{2+} deficiency. Douglas & Rubin (1963) found that when the adrenal medulla was perfused for a period of 20 min with a Ca^{2+}-free

solution, the restoration of Ca^{2+} to the solution caused an immediate release of catechol amines. They suggested that during the period of Ca^{2+}-depletion, Ca^{2+} was set free from its binding sites in the chromaffin cell membrane with the result that this membrane became more permeable to Ca^{2+}. When the Ca^{2+} was restored it was able to enter the cell immediately to release catechol amines.

TABLE 3. Effect of Ca^{2+} concentration on the increase in rate of isolated atria caused by noradrenaline in varying concentrations

Noradrenaline concentration	Percentage change in rate Ca^{2+} concentration (mM)		
	2·2	6·6	13·2
2×10^{-6}	21	10	9
3×10^{-6}	66	113	46
4×10^{-6}	22	40	.
4×10^{-6}	69	97	.
4×10^{-6}	32	60	.
10^{-5}	152	62	.
10^{-5}	133	142	44
Mean	71	75	33

In the previous paper (Burn & Gibbons, 1964) we attempted to demonstrate a similar occurrence in the isolated ileum. Replacement of Locke's solution with Ca^{2+}-free Locke's solution for the short period of 2 min slowed the rhythm and usually diminished the contractions, but on replacing the Ca^{2+}, the rhythm was at once restored fully. If however Ca^{2+}-free Locke's solution was put in the bath for 20 min then, on readmitting Ca^{2+}, the resumption of the pendular movements was inhibited and began again very gradually. We thought that this inhibition might be due to entry of Ca^{2+} into the fibres and to the release of noradrenaline. We have now tested this by comparing normal strips of ileum with strips taken from rabbits treated with reserpine to remove the noradrenaline.

In the normal strip (Fig. 3*a*) replacement of Ca^{2+} after 20 min depletion had no effect during 1 min, and then the rhythm slowly began again. In the strip from the rabbit treated with reserpine, the rhythm began again almost as soon as the Ca^{2+} was replaced (Fig. 3*b*). We compared four strips from normal rabbits with four strips taken from rabbits treated with reserpine, and determined the time from the replacement of Ca^{2+} after 20 min depletion to the point at which the amplitude had fully recovered. The times for the normal rabbits were 3·1, 3·0, 3·1 and 3·8 min. The times for the rabbits treated with reserpine, 1·5, 0·125, 0·4 and 1·5 min, were all shorter than the shortest time for a strip from a normal rabbit. The mean for the normal strips was 3·3 min while that for reserpine-treated strips was 0·88 min. This evidence supported the suggestion that the slow resumption of rhythm in the normal strips was due to the release

of noradrenaline, and that calcium depletion for 20 min resulted in a loss of Ca^{2+} from binding sites in the membrane of the post-ganglionic fibre, so that Ca^{2+} when restored could enter the fibre and release noradrenaline.

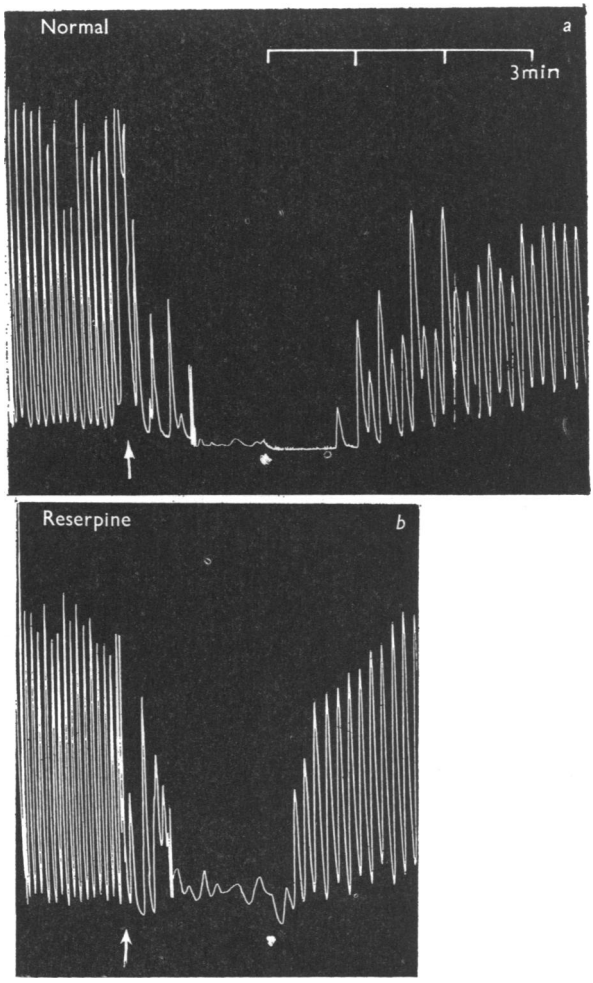

Fig. 3. In the upper panel (3*a*) a normal strip of rabbit ileum. From the arrow to the dot calcium was removed. At the dot it was added to make 4 mM. Note the elapse of 1 min before there was any beginning of the rhythm and the slow rise thereafter. In the lower panel (3*b*), a strip of ileum from a rabbit treated with reserpine. Note that the rhythm began almost at once after the addition of calcium.

Action of guanethidine. We studied the effect of raising the Ca^{2+} concentration in the Locke's solution surrounding the isolated ileum in which the inhibitory action of post-ganglionic stimulation was almost blocked by guanethidine. A result is shown in Fig. 4, in which control observations

were made giving 400 shocks at 5/sec, 20/sec and 80/sec as shown in the top panel. Guanethidine 10^{-6} was then added to the bath, and the developing block is shown in the two middle panels. This block was first evident at 5/sec. In the lower panels, the Ca^{2+} concentration was raised to 4·4 mM (on the left) and then to 6·6 mM (on the right). The rise of Ca^{2+} concentration overcame the guanethidine block, and this reversal of the block was greatest for the lowest frequency.

In experiments in which the guanethidine concentration was 4×10^{-6}, and in which the block of stimulation was complete, there was no reversal of the block when the Ca^{2+} concentration was raised to 6 mM.

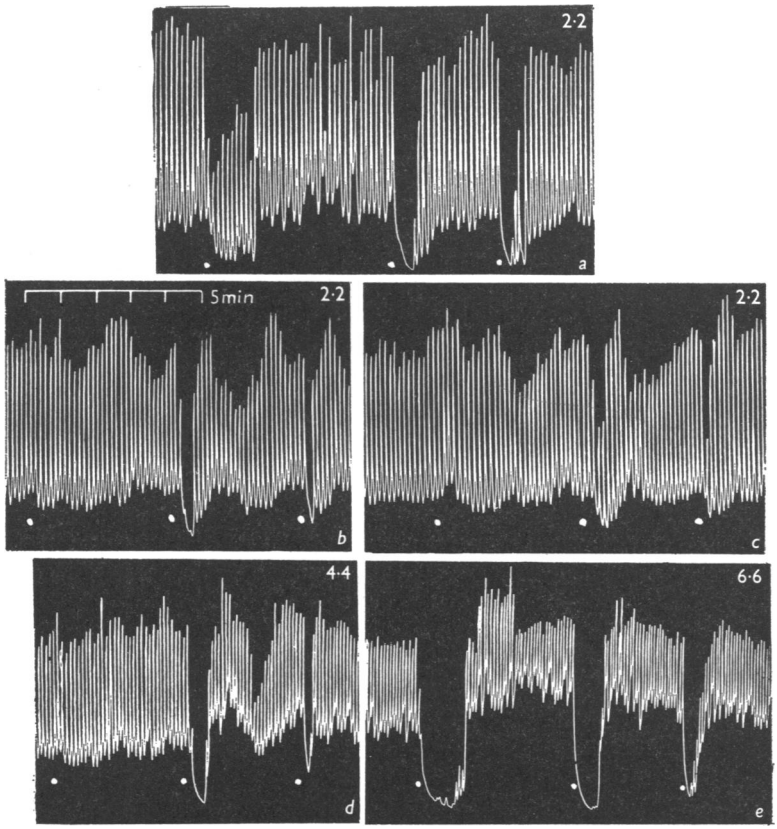

Fig. 4. Rabbit ileum showing inhibitory responses to stimulation with 400 shocks at 5/sec, 20/sec and 80/sec. Top panel shows control responses. Middle panel shows responses after the addition of guanethidine 10^{-6}. In the bottom panel the calcium was raised to 4·4 mM (on the left) and to 6·6 mM (on the right). Note the reversal of the block caused by guanethidine, particularly for the frequency of 5/sec.

DISCUSSION

These results show that there is a close similarity between the release of catechol amines from the adrenal medulla by ACh and the release of noradrenaline from the post-ganglionic fibre by ACh. In the first place we have evidence that the actual release in both cases is due to the entry of calcium. Adding extra calcium to the Locke's solution perfusing the adrenal gland does not cause an output of catechol amines, and adding extra calcium to the Locke's solution bathing a strip of ileum does not cause inhibition. But if in either case there is a period of 20 min in which the Locke's solution contains no calcium, then when calcium is replaced, there is an output of catechol amines from the adrenal gland, and there is inhibition in the ileum. Since this inhibition in the ileum is absent when the ileum is taken from a rabbit treated with reserpine, the evidence strongly supports the view that the inhibition is due to the release of noradrenaline. The suggestion has been made by Douglas & Rubin (1963) that the period of 20 min in which the adrenal medulla is perfused without calcium must be a period in which calcium becomes detached from binding sites in the chromaffin cell membrane so that the membrane is then much more permeable to calcium. When the calcium is replaced the calcium then enters the chromaffin cell and releases catechol amines. We suggest that the observations concerning the release of noradrenaline from the post-ganglionic fibre by calcium after a period of calcium absence are to be explained in exactly the same way.

The action of ACh in increasing the rate of the isolated atria in the presence of hyoscine is a nicotine-like action of ACh, which nicotine also possesses. Burn & Rand (1958) showed that the increase in rate caused by nicotine was not seen in preparations from animals treated with reserpine, and was therefore due to release of noradrenaline. Goodall (1951) showed that the noradrenaline in the heart was present in the post-ganglionic sympathetic fibres, since it was reduced to a very small amount when the post-ganglionic fibres degenerated.

Thus a second point of similarity between the events in the adrenal medulla and those in the post-ganglionic fibre is that ACh causes a release of catechol amines in the one, and of noradrenaline in the other, and the release in both places is dependent on the calcium concentration.

The suggestion was made by Ferry (1963) that the release of noradrenaline by ACh in the spleen was not a direct effect of ACh but was mediated by the sympathetic fibres. He found that the injection of ACh into the splenic artery caused antidromic impulses to pass backward along the splenic nerves, and therefore concluded that impulses would be conducted into the spleen also. However, it has recently been shown

by Hertting & Widhalm (1965) that when the spleen is perfused with a solution containing bretylium in a concentration from 2×10^{-6} to 10^{-5}, the effect of stimulating the splenic nerves in releasing noradrenaline is blocked, while the effect of ACh is still present. If the concentration of bretylium is raised to 5×10^{-5}, then the effect of ACh is also blocked. This evidence makes clear that the effect of ACh is not mediated by the post-ganglionic fibres, and is exerted independently.

These considerations show that the parallel between the action of ACh in the adrenal medulla and in the post-ganglionic fibre is very close indeed. In both places ACh can act directly to allow the entry of calcium (into the chromaffin cell in the one place, and into the post-ganglionic fibre in the other) with the result that catechol amines are liberated in the one, and noradrenaline in the other.

We know since the work of Feldberg & Minz (1933) that ACh is the transmitter of impulses from the splanchnic nerves to the adrenal medulla. Burn & Rand (1959) have given reasons for thinking that in the post-ganglionic fibre there is a cholinergic link in the release of noradrenaline, and that the post-ganglionic fibre, like the splanchnic nerve, liberates ACh in the first place.

SUMMARY

1. The finding that the response to stimulating post-ganglionic fibres depends on the external calcium concentration has been confirmed.

2. Evidence is given that acetylcholine, acting in the presence of atropine or hyoscine, releases noradrenaline from the post-ganglionic terminations in the atria, and that the amount released also depends on the external calcium concentration.

3. The acceleration of the atrial rate by tyramine or by noradrenaline does not appear to depend on the calcium concentration.

4. Evidence is given to show that when the post-ganglionic fibre is immersed for 20 min in a calcium-free solution, restoration of the calcium results in liberation of noradrenaline. This occurrence is similar to that seen in the adrenal medulla, and it is interpreted similarly: during the period in which calcium is absent, the membrane becomes more permeable and when calcium is restored it enters the fibre and releases noradrenaline.

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