Acetylcholine Tachyphylaxis in Isolated Rabbit Atrium and Its Relation to Norepinephrine Stores

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Abstract ☐ Large concentrations of acetylcholine, in the presence of atropine, are known to produce positive inotropic and chronotropic responses in isolated, spontaneously beating rabbit atria. This response is due to release of norepinephrine from atrial tissue. After repeated concentrations of acetylcholine (100 mcg./kg.), in the presence of atropine (3 mcg./kg.), were added to the bath fluid at 10-min. intervals, the usual stimulatory responses to acetylcholine were not detected; *i.e.*, the tissue was tachyphylactic to the actions of acetylcholine. If acetylcholine, in the presence of atropine, was given at intervals of more than 20 min., tachyphylaxis could not be observed. To investigate a possible link between acetylcholine tachyphylaxis and a decrease in the amount of norepinephrine available for release by acetylcholine, the atria were exposed to norepinephrine after acetylcholine tachyphylaxis was produced. Norepinephrine was allowed to remain in contact with tachyphylactic atrial tissues for 5 min. before being washed out. Acetylcholine now elicited its usual stimulatory effect. Epinephrine was also capable of restoring the response of tachyphylactic atria to acetylcholine. Atria excised from rabbits that were pretreated with reserpine did not respond to either acetylcholine or tyramine. When these preparations were incubated with norepinephrine, there was no return of the stimulatory response to acetylcholine, although the response to tyramine was restored.

Keyphrases ☐ Acetylcholine tachyphylaxis—isolated atrium, rabbit ☐ Norepinephrine stores, relationship—acetylcholine tachyphylaxis ☐ Reserpine effect—acetylcholine, tyramine activity ☐ Atropine effect—acetylcholine tachyphylaxis

Tachyphylaxis is the phenomenon in which repeated administration of a drug to a test system at short time intervals leads to progressively smaller and smaller responses induced by the drug (1). Indirectly acting sympathomimetic amines such as amphetamine (2), tyramine (3), and ephedrine (4), as well as cocaine (5), owe their adrenergiclike effects to release of endogenous norepinephrine from the test tissue, and the tissue develops tachyphylaxis due to the gradual depletion of essential norepinephrine stores (6).

It has been demonstrated that large doses of acetylcholine (ACh), when given either in the presence or absence of atropine, released norepinephrine (NE) from isolated cardiac tissue (7, 8). This release has also been shown for indirectly acting sympathomimetic amines (9). Because of this similarity between the indirectly acting sympathomimetic amines and ACh, the authors attempted to produce tachyphylaxis to the positive inotropic and chronotropic actions of ACh on the isolated spontaneously beating rabbit auricles.

It has also been demonstrated by Cowan *et al.* (1, 3, 4) that after production of tachyphylaxis to indirectly acting sympathomimetic amines, an exposure of the tachyphylactic tissue to NE restores, to a lesser or greater degree, the responses to subsequent challenges with the drug. Therefore, the effect of an exposure of ACh–tachyphylactic atrial tissue to NE and epinephrine (E) was tested. Additionally, the absence of responses to ACh in the tachyphylactic preparation was compared to the absence of response to ACh observed in atrial tissues taken from rabbits that were pretreated with reserpine.

It has been proposed (6, 10, 11) that cardiac tissue has a number of storage compartments for NE and that the different compartments may be induced to release this neurohumor by different stimuli. In these studies, the authors also attempted to differentiate between compartments from which ACh and tyramine release NE.

METHODS

All experiments were performed on the isolated, spontaneously beating rabbit atria. Male albino rabbits, weighing 1.2–2.3 kg., were sacrificed by a blow to the nape of the neck. The heart was then rapidly excised and placed in oxygenated Locke solution of the following composition (in g./l.): NaCl, 9.0; KCl, 0.42; $CaCl_2$ (dihydrate), 0.24; $NaHCO_3$, 0.5; and dextrose, 2.0. The right and left atria were separated together from the ventricles and, after washing and removal of excess tissue, were mounted in a 40-ml. organ bath filled with Locke solution at 30 ± 1° through which 100% O_2 was continuously bubbled.

One end of the atrial preparation was attached to a Starling heart lever, loaded to exert a tension of 1 g., which recorded the amplitude of atrial contractions on a smoked paper kymograph. The rate of contraction was recorded using a Thorp impulse counter calibrated so that 1 mm. of deflection was equal to one contraction.

A 1-hr. period was allowed for equilibrium before the experimental protocol was initiated.

The animals used in the studies requiring pretreatment with reserpine were given 5 mg./kg. i.p. 20–24 hr. prior to the experiment.

Statistical analysis of the experimental data was carried out as described by Batson (12). A probability of 5% ($p = 0.05$) was considered maximum for statistical significance.

The following drugs were used in this study: acetylcholine bromide,[1] *l*-adrenaline (*l*-epinephrine) bitartrate,[1] atropine sulfate,[2] levarterenol (*l*-NE) bitartrate monohydrate,[3] tyramine hydrochloride,[4] and reserpine USP[5] (ampuls, 2.5 mg. base/ml.).

The concentrations of drugs, with the exception of reserpine, were expressed in terms of their respective salts. The concentrations of the solutions were such that it was not necessary to add more than 0.3 ml. of the drug solution to the bath fluid.

RESULTS

Dose–response curves to ACh were determined by exposing the atria to progressively increasing concentrations of ACh. ACh was added to the organ bath 3 min. after an addition of atropine (3 mcg./ml.); each addition of ACh was followed by a 30-min. period with numerous washes prior to repetition of the described procedure.

The dose–response curve to ACh (Fig. 1) shows that the force and rate of atrial contraction increased in a linear fashion with increasing concentrations of ACh up to 100 mcg./ml., after which the change in rate decreased with increasing doses of ACh until a negative chronotropic response became apparent.

[1] Eastman Organic Chemicals, Rochester, N. Y.
[2] Merck & Co., Rahway, N. J.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Mann Research Laboratories, New York, N. Y.
[5] Serpasil, CIBA Pharmaceutical Co., Summit, N. J.

Table I—Responses of Isolated Rabbit Atria (n = 8) to ACh (100 mcg./ml.), in the Presence of Atropine (3 mcg./ml.), before and after the Production of Tachyphylaxis

Parameters Measured	Control Values	Change from Control Values: Control Response to ACh ± *SE*	Change from Control Values: Response to ACh ± *SE* after Production of Tachyphylaxis
Force[a]	22 ± 2.1	+9 ± 1.0	−5 ± 1.3[c]
Rate[b]	126 ± 7.8	+36 ± 4.2	−12 ± 2.4[c]

[a] Mean force in mm. [b] Mean rate in beats/min. [c] Comparison of responses to ACh before and after tachyphylaxis; p = 0.05 or less.

The stimulatory response to ACh (100 mcg./ml.), in the presence of atropine (3 mcg./ml.), was characterized by a very short latent period (2–4 sec.) followed by a marked stimulation of both force and rate. After reaching their maximum, the parameters started to return to control levels; when this occurred the bath fluid was exchanged repeatedly. These responses could be reproduced many times when ACh and atropine were given at 30-min. intervals. Results from all subsequent experiments are compared with these described responses.

Production of Tachyphylaxis to ACh—For the demonstration of true tachyphylaxis, certain criteria must be satisfied. The same concentration of ACh must be administered at equal time intervals, and all measured parameters must be allowed to return to predrug levels prior to the next administration of the challenging agent. A concentration of 100 mcg./ml. of ACh was used and was preceded for 3 min. by atropine (3 mcg./ml.). An interval of 10 min. between ACh responses was found to be optimal for the production of tachyphylaxis. When the time interval between responses was prolonged to 20 min. or longer, tachyphylaxis could not be produced. The response to ACh lasted about 1 min.; thus there was a sufficient time interval between drug additions to allow all parameters to return to control levels.

Using these experimental conditions, complete or nearly complete tachyphylaxis was produced following the administration of four doses of ACh. The response of atria to the administration of ACh was considered to be tachyphylactic when the preparation no longer produced an augmentation of atrial contractions. In most experiments, augmentation was replaced by depression. Table I summarizes the measurements obtained in the production of tachyphylaxis.

When the aforementioned schedule of drug additions was continued throughout the duration of the experiment, a spontaneous return of the normal positive inotropic and chronotropic response to ACh could not be observed. However, if after the production of tachyphylaxis the administration of ACh was discontinued for a minimum of 35–45 min., a spontaneous return of the normal response to ACh was observed.

After the establishment of tachyphylaxis to ACh, the indirectly acting sympathomimetic amine, tyramine (5 mcg./ml.), was added to the atrial bath and produced its characteristic augmentation of atrial contractions. Thus, the authors have found no evidence of cross tachyphylaxis between the stimulatory actions of ACh and tyramine.

Reversal of Tachyphylaxis to ACh by Catecholamines—Since the stimulatory response of atria to additions of ACh is the result of a release of NE (7), the development of tachyphylaxis may be attributed to a decrease in the availability of releasable NE and, therefore, should be modified, or perhaps reversed, by exposure to this amine.

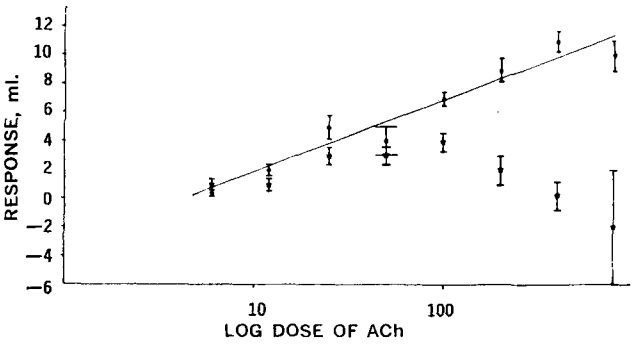

Figure 1—*Dose–response curve to ACh in the presence of atropine (3 mcg./ml.). Key:* ⟊, *force* ± SE; ⟊, *rate* ± SE.

Tachyphylaxis was produced in the manner previously described. Upon production of tachyphylaxis, the atria were incubated with NE (0.025 mcg./ml.) for 5 min. The NE was then washed out and the response to ACh was tested in the presence of atropine. The NE incubation procedure did not interrupt the 10-min. interval between additions of ACh. Table II demonstrates the effect of NE incubation upon the tachyphylaxis to the stimulatory response to ACh. As can be seen, restoration of the normal positive inotropic and chronotropic response was attained. Figure 2 is a tracing of a typical experiment of this type.

Since incubation with NE proved to be effective in restoring the stimulatory response to ACh in tachyphylactic atria, another catecholamine, E (0.025 mcg./ml.), was employed under similar conditions. Table II shows the results of these experiments. The response of the atria to ACh, following E incubation, was restored to control levels as it was following incubation with NE.

Effect of Other Agents upon the Response of Atria to ACh—Atria were excised from rabbits that had received reserpine[6] (5 mg./kg. i.p.) 24 hr. prior to the experimental procedure. In these preparations, the addition of ACh in the presence of atropine failed to produce a positive inotropic and chronotropic response. This was instead supplanted by a negative inotropic and chronotropic response. When reserpine-pretreated atria were incubated with NE (0.025 mcg./ml.) in the manner previously described, there was no restoration of the normal stimulatory response to ACh as was observed in the tachyphylactic atria. Table III summarizes the results of these experiments. However, a restoration of the response to tyramine in reserpine-pretreated atria could be demonstrated following incubation with NE.

DISCUSSION

The positive inotropic and chronotropic effect of ACh on the atropine-pretreated atria is a well-known phenomenon (13–15). Hoffman *et al.* (14) and Middleton *et al.* (16) have proposed that this atrial stimulation is a result of release of an epinephrinelike substance from cardiac tissue, either due to ganglionic stimulation or release from chromaffin tissue. The investigations of Richardson and Woods (7) and of Angelakos and Bloomquist (8) have established that this epinephrinelike substance is, in fact, NE. Thus, one must come to the conclusion that ACh exerts its stimulatory action *via* release of NE in the atropine-pretreated isolated rabbit atria.

Table II—Effect of Incubation with Catecholamines upon Responses of Tachyphylactic Isolated Rabbit Atria to ACh (100 mcg./ml.) in the Presence of Atropine (3 mcg./ml.)

Parameters Measured	Control Values	Catecholamine, CA, 0.025 mcg./ml.	Change from Control Values: Tachyphylactic Response to ACh before CA ± *SE*	Change from Control Values: Control Response to CA ± *SE*	Change from Control Values: Response to ACh after CA ± *SE*
Force[a]	18 ± 1.2	Norepinephrine, n = 6	−7 ± 2.5	+8 ± 1.8	+7 ± 1.4[c]
Rate[b]	138 ± 4.2		−6 ± 1.8	+30 ± 10.8	+18 ± 4.8[c]
Force[a]	20 ± 0.9	Epinephrine, n = 5	−6 ± 2.4	+6 ± 2.1	+8 ± 1.1[c]
Rate[b]	114 ± 8.4		−18 ± 8.4	+30 ± 10.8	+30 ± 9.6[c]

[a] Mean force in mm. [b] Mean rate in beats/min. [c] Comparison of responses to ACh before and after CA; p = 0.05 or less.

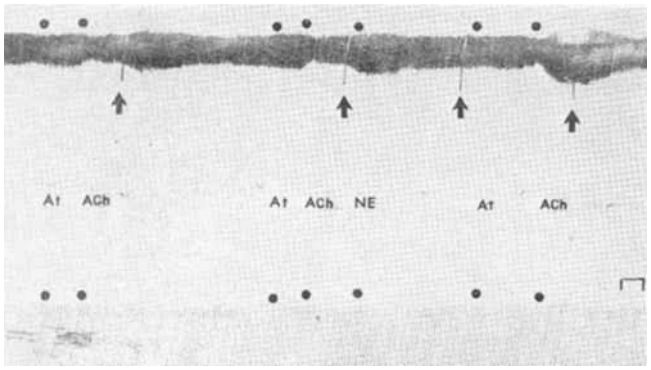

Figure 2—*Effect of NE upon tachyphylaxis to large doses of ACh in isolated rabbit atria.*

The upper tracing measures contractile force and the lower tracing depicts atrial rate. The horizontal bracketed line represents 1 min. The drugs were added at the black dots; ACh, 100 mcg./ml.; At, atropine, 3 mcg./ml.; NE, 0.025 mcg./ml. At the arrows the bath fluid was exchanged three times while the kymograph was arrested. The first two doses of ACh illustrate the response of the tachyphylactic atria to this drug. The third dose of ACh illustrates the response after incubation with NE.

While some experimental evidence (17–19) supports the proposal that ganglionic stimulation is the mechanism by which ACh exerts its effect, results of other studies suggest that this mechanism is not an adequate explanation. Anatomical and histological studies have shown that, although intrinsic ganglia are present in the atria, these structures are all parasympathetic and not sympathetic (20–22). Hirsch *et al.* (23) and Cooper (24), using cardiac transplantation techniques to ensure complete denervation, indicate that ganglionic stimulation by ACh is an unlikely mechanism for the positive inotropic and chronotropic effect in such preparations. Studies using the formaldehyde vapor-condensation technique have shown that there is little or no fluorescence in the atria after sympathetic denervation (25). Lee and Shideman (26), using cat papillary muscle, have demonstrated that, in atropine-pretreated preparations, large doses of ACh produced positive inotropic and chronotropic responses. Careful histological studies of these papillary muscles have shown no ganglia to be present; only sympathetic nerve endings were observed.

It is extremely unlikely, therefore, that ACh produces its stimulatory action by acting through the stimulation of intrinsic sympathetic ganglia in the atria. It is more likely that it is acting by the release of NE from the postganglionic neurons. This may be accomplished in a manner similar to that described by Burn and Rand (27).

In view of this evidence and present experimental data, it would seem more meaningful and correct to characterize the actions of ACh and other nicotinic agents on the atria as indirect actions rather than nicotinic effects. The term nicotinic implies sympathetic ganglionic stimulation, and this is clearly not applicable to atria.

Cowan *et al.* (3, 4), Maengwyn-Davies (28), and Maengwyn-Davies *et al.* (2, 29) have substantiated the general theory of Koppanyi (30) that drugs whose action depends upon the liberation of biogenic substances will show tachyphylaxis due to the gradual depletion of releasable biogenes. Indirectly acting ACh meets these stipulations and is indeed tachyphylactogenic on the rabbit atrium, as are the other indirectly acting atrial stimulants.

Incubation of the atria with NE restores the usual indirect response to ACh, which is lost during tachyphylaxis, presumably by replacement of essential catecholamines. The authors have also shown that E, as well as NE, can reverse the tachyphylaxis to ACh. The restoration of the response to ACh by E is in full agreement with the observations of Potter (31) who showed that E and NE are equally well bound to isolated storage granules. Therefore, in these experiments, E may have acted as a "false transmitter." Another explanation might be that of Raab and Gigee (32) who proposed that E may displace some NE from storage sites and then be demethylated to NE.

There seems to be some question as to whether there is a depletion of NE when the stimulatory response to ACh is absent. Using somewhat different methods, Torchiana and Angelakos (33) have demonstrated that when the stimulatory response to ACh in atropinized preparations was absent, there was no significant depletion of NE from the atrial tissue. Furthermore, they have shown that ACh administered to nonatropinized cardiac tissue causes a liberation of NE, accompanied by an increase rather than a decrease in cardiac catecholamines, without, of course, a positive inotropic and chronotropic response (8). This failure to demonstrate a depletion of NE could be interpreted in two ways. First, these authors administered acetylcholine at 20-min. intervals. At this time interval, there could be a restoration of liberated catecholamines by the atrial tissue since the present authors have shown that ACh administered at 20–30-min. intervals failed to produce tachyphylaxis. Secondly, the ACh releasable store may be small, and depletion of this store would not give a significant reduction in the total catecholamine content of the atria. These proposals may gain support in the observations that the atria is still responsive to tyramine while tachyphylactic to ACh. Moreover, there must be some depletion of NE, as indicated by the observation that tachyphylaxis to ACh is reversed by incubation of the atria with this neurohormone.

In atria excised from reserpine-pretreated rabbits, all agents acting by the release of NE are ineffective. The authors have shown this to be true for ACh, just as it has been shown for nicotine (34), DMPP (35), and tyramine (3). This lack of response in all cases is most probably a result of the depletion of NE by reserpine (36). In the studies reported here, however, atria from reserpine-pretreated rabbits which showed no indirect stimulating response to ACh could not be made to respond upon incubation with catecholamines while the response to tyramine was reinstated. This difference in behavior of indirectly acting ACh and of tyramine and DMPP in atria from reserpine-pretreated animals could be explained by the depressant action of ACh (Table III) and the lack of such depressant action by tyramine. The depressant effect of ACh may be due to stimulation of receptor sites not occupied by atropine, resulting in atrial depression (37).

The lack of cross tachyphylaxis between ACh and tyramine, and the restoration of the response to tyramine but not to ACh in reserpine-pretreated atrium, indicate that these two agents release NE from different stores. When the ACh-releasable store is apparently depleted, the tyramine-releasable store is still unaffected.

The results seem to indicate that there is no uptake of NE by the ACh-releasable compartment in reserpinized atria. If NE was taken up, some would probably be released by ACh, with the result (which is not observed) being a reduction or reversal of the depressant response to ACh. The reserpine-pretreated atria was, however, capable of taking up and releasing NE, as indicated by the restoration of the atrial response to additions of tyramine.

The contrast between the effects of reserpine pretreatment and tachyphylaxis on the atrial response to ACh is quite perplexing. In the case of tachyphylaxis, the negative inotropic and chronotropic effects of large, oft-repeated doses of ACh can be easily converted into atrial stimulation by incubation with catecholamines; thus the tachyphylaxis can be explained by the loss of essential catecholamines. These ACh-releasable catecholamines probably represent only a small fraction of the total catecholamines, because tachyphylaxis is reversed by allowing the atrial preparation to stand without further additions of ACh for 35–45 min.

Table III—Effect of Incubation with NE (0.025 mcg./ml.) on Responses of Reserpine-Pretreated[a] (n = 5) Isolated Rabbit Atria to ACh (100 mcg./ml.) in the Presence of Atropine (3 mcg./ml.)

Parameters Measured	Control Values	Change from Control Values: Tachyphylactic Response to ACh before NE ± *SE*	Change from Control Values: Control Response to NE ± *SE*	Change from Control Values: Response to ACh after NE ± *SE*
Force[b]	20 ± 1.1	−8 ± 2.6	+13 ± 1.1	−8 ± 3.1
Rate[c]	114 ± 10.3	−24 ± 3.0	+42 ± 12.0	−24 ± 5.4

[a] Reserpine, 5 mg./kg. i.p., 24 hr. before sacrifice. [b] Mean force in mm. [c] Mean rate in beats/min.

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Effect of Sex on Penicillin Blood Levels in Dogs

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Abstract ☐ Studies concerned with the oral absorption characteristics of dicloxacillin from various pharmaceutical formulations in beagle dogs suggest that the female of this species shows consistently higher and more prolonged blood serum levels of the antibiotic than the corresponding male. An investigation was initiated to determine the extent of this phenomenon with respect to sodium dicloxacillin monohydrate and other penicillins. The results of these studies suggest that the monobasic penicillin molecules show blood serum level variations after oral administration that are related to the sex of the animal, while a similar correlation does not appear to exist for the amphoteric penicillins. Determination of the biological half-life of the various penicillins in the male and female after intravenous administration indicates that no sexual differences occur with respect to the disappearance of active drug from the blood.

Keyphrases ☐ Penicillin blood levels—dogs ☐ Sex effect, dogs—penicillin blood levels ☐ Blood levels, half-life, corticosteroids—sex effect, dogs ☐ Microbiological test method—analysis

Minor sexual differences in drug response among the animals of a species are frequently encountered in toxicity experiments. For example, L-thyroxin produces a more pronounced depressant effect on weight gain in male than in female rats (1), and the toxicity of hypoglycemic agents is enhanced in female and male rats pretreated with diethylstilbestrol (2). These differences are sometimes quite significant. For example, the antibiotic acetoxycyclohexamide was shown by Pallotta *et al.* (3), in acute and subacute tests, to be about four times as toxic for young female rats as it is for males. In many cases, these differences in response between the sexes can be traced to differences in enzyme activities and rate of metabolism. Male rats metabolize hexobarbital much faster than do females, and the average sleeping time of the male after receiving the drug is only about one-fourth that of the female (4, 5). Recently, in a report by Kernohan and Todd (6), it was suggested that women bleed more readily than men during heparin therapy. A similar conclusion, that women have a higher risk of bleeding with heparin than do men, was made from the studies of Jick *et al.* (7).

The results obtained in a series of investigations on the effect of dosage-form variables on biological avail-