Norepinephrine Sensitivity and Desensitization of Cultured Single Vascular Muscle Cells

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Summary. Isolated single vascular muscle cells were used for studies of the inherent norepinephrine sensitivity and the conditions important for detecting highest norepinephrine sensitivity. Rat vascular muscle cell contractions were quantitated from spontaneous contractions during pulse applications of the drugs, with a time course designed to simulate norepinephrine release in situ. Isolated vascular muscle cells showed a sensitivity to norepinephrine two orders of magnitude greater than those found from isolated intact blood vessels. Associated with the high sensitivity, there was a marked reduction in the response to a second application of norepinephrine or phenylephrine. The reduced second response appears to result from desensitization that is more pronounced in cells that have been exposed to only trace concentrations of catecholamines. These data appear to suggest that there might be continuous suppression of transmitter sensitivity that occurs as a result of transmitter exposure, and possibly cell-to-cell associations. Desensitization would at first severely limit the response to norepinephrine by reducing or eliminating the response to prolonged exposure or a second dose. Thereafter, a lessening of the desensitization process on continuous exposure to catecholamines would be the result, in part, of lowered sensitivity and, in part, of a smaller desensitization response. This process would continuously modulate norepinephrine sensitivity, based on the frequency and extent of stimulation, and we have called it the theory of physiological desensitization. (Circ Res 50: 627-632, 1982)

ONE of the most important influences to determine the magnitude of a blood vessel response to norepinephrine (NE) is the sensitivity of the vascular muscle cells to adrenergic stimulation. Whereas we normally take for granted that the sensitivity will be rather constant for a blood vessel under normal conditions, there are important identified influences of blood vessel innervation on NE sensitivity (Fleming et al., 1973). Strong evidence that arrangement of neuronal and other cells within the blood vessel wall are important determinants of sensitivity is provided by our recent reports of very high sensitivity to NE in both cardiac and vascular muscle cells studied under culture conditions (Hermsmeyer, 1976; Marvin et al., 1979; Abel et al., 1980; Hermsmeyer and Aprigliano, 1980). In this study, we have further explored the inherent sensitivity of vascular muscle to adrenergic stimulation by use of isolated single cells and small groups of cells from neonatal rats.

Cardiac and vascular muscle cells from young animals appear to have a very high NE sensitivity because they have not previously been exposed to very high levels of catecholamines, which probably avoids the physiological desensitization that appears to control the NE sensitivity (Hermsmeyer and Robinson, 1977). Highly sensitive cultured isolated single cells reveal the desensitization process as being sufficient to cause nearly complete loss of catecholamine sensitivity, which probably explains the early reports (Sperelakis and Lehmkühl, 1965) of no sensitivity to autonomic agents in cultured cardiac muscle. In cells where desensitization has not yet occurred, it is very important to apply drugs such that they reach the muscle cells in full concentration in a fraction of a second, rather than allowing diffusion, requiring seconds or minutes, during which desensitization greatly attenuates the response (Hermsmeyer and Robinson, 1977).

In the present experiments, we have studied adrenergic stimulation which increases spontaneous contraction frequency of cultured vascular muscle cells. These experiments allow quantitation of contraction frequency before, during, and after exposure of rat vascular muscle cells to the adrenergic agonists, norepinephrine and phenylephrine. We have applied these drugs in microliter quantities to a small, specially designed laminar flow chamber which allows highly reproducible quantitation of vascular muscle contraction frequency.

Methods

Cell cultures were prepared from the lower thoracic portion of azygous veins (0.5 mm X 7 mm) of neonatal rats (0–4 day) from our inbred colony of spontaneously hypertensive rats (SHR) and their Kyoto-Wistar normotensive controls (WKY). Cell culture preparations were prepared, as previously described (Marvin et al., 1979), using collagenase pre-soak (without stirring) followed by trypsinization, and were studied from 2–14 days later. We believe azygous venous muscle cells, which contract rapidly and at
higher spontaneous frequencies than aortic muscle cells, are the same as the fast contraction population previously described (Hermsmeyer, 1979). The most important consideration was limiting enzyme exposure by using low enzyme concentrations and removing cells frequently to protect already dissociated cells. CV3M, our present culture medium, is based on MEM (minimum essential media, Eagle) adjusted for maintaining pH 7.4 during culture by inclusion of 20 mM HEPES buffer (as dry powder) in a ratio of NaHEPES to HEPES acid that gives pH 7.40 at 37° and 16 mM NaHCO₃. CV3M consists of 83% MEM, 15% horse serum, and 2% l-glutamate (final concentration 4 mM). The other significant change of our previous methods has been the exclusive use of potassium glutamate solution (KG) for all of the dissociation steps with trypsin and collagenase. The potassium glutamate solution seems to reduce the degree of potassium loss and chloride gain by the cells during the exposure to the enzymes. KG solution consists of (in mM): K-glutamate, 135; NaHCO₃, 16; NaH₂PO₄, 0.5; HEPES (pH 7.4 at 37°), 20; dextrose, 16.5; and phenol red, 0.014. Collagenase exposure was 3 mg/ml for 30 minutes and trypsin exposure was 0.75 mg/ml for 6-8 15-minute periods with gentle (1-2/sec) stirring.

Quantification of the responses to NE and phenylephrine was carried out by counting contractions, either directly on the microscope, or from filmed records of contractions (Leicina Special cine film), or by photomultiplier recording and display on an oscilloscope or Brush paper chart recorder. In each case, we counted the spontaneous contractions for a 1-minute period followed by responses to phenylephrine or NE during each 15-second interval after addition. The peak response was taken as the datum point, which always occurred during the first seconds after arrival of the drug at the cells. Counting contractions directly on the microscope gave exactly the same mean result and variability as recording the data and analyzing films or photomultiplier traces. Because of desensitization, only one coverslip of cells was used for a single response, except in the last figure which demonstrates the desensitization. All experiments were carried out with a Leitz Diavert (inverted microscope), using enhanced contrast optics (either phase contrast or K contrast). The laminar flow chambers are the 1980 version of the one described previously (Hermsmeyer and Robinson, 1977) produced by Jim's Instrument Manufacturing. Drugs were added as 10-, 20-, or 30-μl pulses by Eppendorf pipette at the upstream end with continuous perfusion in the laminar flow chamber. Perfusion solution was ISM, which consists of (in mM): NaCl, 130; NaHCO₃, 16; NaH₂PO₄, 0.5; KCl, 4.7; CaCl₂, 1.8; MgCl₂, 0.41; MgSO₄, 0.41; HEPES (pH 7.4 at 37°), 13; and dextrose, 5.5. NE and phenylephrine were prepared as 1 mM stock solutions immediately before use and diluted to final plotted concentrations in ISM immediately before application. Increases in contraction frequency caused by the mechanical artifact of adding the small pulse of fluid were tested during each experiment by the addition of ISM and typically were less than 5%. All experiments were carried out under thermoregulated control at 36-37°C by use of a heat exchange bottle and a sub-stage heater on the microscope (Jim's Instrument Manufacturing). Application of the drugs to the

**Figure 1. Light microscopic appearance of vascular muscle cells studied in small groups or as single muscle cells for the functional data.** Magnifications are at 300 X. Panels A and C represent phase contrast microscopy, while panels B and D represent K interference contrast appearance. Notice that there are several different cell morphologies involved, and the contracting cells are not a completely homogeneous population.
cells reached full concentration within 100 msec, as verified by the measurement of the time course of light transmittance or fluorescence during the application of 30 μM trypan blue or 1 μM 6-carboxyfluoresceinate dye with a microscope photometer (Leitz MPV Compact). The dye increased from 0 to maximum concentration in less than 100 msec when applied exactly as the norepinephrine and phenylephrine were applied. The modal time to peak dye concentration was 40 msec for 100 such determinations in the five different laminar flow chambers used.

Drugs used in these experiments were l-norepinephrine hydrochloride and phenylephrine hydrochloride (both from Sigma Chemical Co.). Statistical comparisons were carried out by group t-test.

Results

The cells were plated in culture to produce low densities that would be suitable for single cell experiments. The morphology of vascular muscles found spontaneously contracting is shown in Figure 1. Both phase contrast (A and C) and K contrast (B and D) show typical living, contracting vascular muscle cells which existed in many configurations. Although it is possible, with experience, to learn which cell shapes are most likely to contract, it is never possible to predict by morphology alone whether a given cell is a muscle cell or is incapable of contraction, either because it is non-muscle or has lost contractile characteristics (excitability or contractile apparatus). The only reliable criterion for identification of muscle cells was the appearance of contractions, either spontaneously or induced by stimulation. Figure 1 illustrates that the contracting cells existed in a number of configurations, and that individual cells or small groups could readily be found for the drug experiments. The type of contractions that were observed is shown in Figure 2, which is an optical record giving the time course of contractions. The contracting cells were attached to glass coverslips, but the contractile apparatus shortened, changing the shape of the cells by thickening the contraction region and therefore changing the light intensity monitored by the photometer. Spontaneous frequency of contractions for a given cell or cell group was reasonably constant, even though the contraction pattern was not nearly so regular as that found in cardiac muscle. On addition of NE, the contraction frequency increased.

A dose-response curve for NE is shown in Figure 3. NE was applied as pulses in the concentrations shown on the ordinate, and arrived instantaneously at the cells with little dilution because of the laminar flow chamber designed for these transmitter sensitivity experiments. The NE reached full concentration in less than 100 msec (see Methods) and would have fallen to <1% of the initial concentration in approximately 15 seconds, conditions we have chosen to reasonably simulate nerve release of NE in the intact vessel. Each cell was used for only one NE concentration because the first exposure desensitized the cells. There was an increase in contraction frequency spanning more than five log units, and—at beyond 1 μM concentrations—the contraction frequency was less

![Optical records of vascular muscle contraction](image-url)
than submicromolar concentrations, probably because the duration of the high contraction frequency was less than the 15-second counting period; a burst of only 10-20 high frequency contractions in 4-6 seconds was followed by a period of quiescence, apparently indicating a maximum response limit of the cells and desensitization.

The dose-response curve to phenylephrine was similar in having both an increasing and decreasing phase, but occurred at lower concentrations (Fig. 4, upper solid curve). Above 1 nM there was a loss of the maximum response, suggesting that desensitization occurred before a peak response could be measured or that the cells limited maximum stimulation. The comparison of NE and phenylephrine sensitivity of isolated cells as compared to intact blood vessels is shown in Table 1. We have also included data from our organ culture experiments, which showed a modest increase in NE sensitivity of the order of magnitude that would be expected for the portal vein, based on denervation supersensitivity. The increase in sensitivity to NE was over two orders of magnitude, compared to the azygous vein freshly removed from rats of the same age. Phenylephrine sensitivity was three orders of magnitude greater than found in the freshly isolated intact azygous vein. Because the azygous vein is a prominent vessel in 2-day-old neonatal rats, a preparation 500 µm in diameter could be isolated and its contraction frequency studied. For this comparison, isolated veins were exposed to agonists under conditions as close to the protocol for cultured cells as possible, except that tension responses were recorded from portal veins because α-agonists do not increase contraction frequency. Portal veins were from adult rats and were not denervated (intact), rapidly denervated in vitro by 6-hydroxydopamine, or in vitro-denervated and organ-cultured for 2 days (Abel et al., 1980). Azygous veins were from 2-day-old neonatal rats, in which they are a prominent vessel.
Discussion

These experiments demonstrate that isolated vascular muscle cells have high sensitivity to adrenergic stimulation—more than 100 times higher than do isolated blood vessel strips. All of the data collected in these experiments was from contracting vascular muscle cells in culture. Although it is much more difficult to succeed in maintaining contractile ability in vascular muscle than in visceral muscle, there have now been reports from four laboratories that the contractions of vascular muscle and responsiveness to drugs can be maintained by special care in preparation (Mauger et al., 1975; Hermsmeyer, 1976; Richter et al., 1976; Harder and Sperelakis, 1979). On the other hand, most of the papers published on cultures of vascular muscle have used preparations that had lost many characteristic features of the vascular muscle cells, including contraction (Chamley-Campbell et al., 1979). Ideally, membrane characteristics of isolated vascular muscle cells should be altered as little as possible by culturing procedures. Based on the current and previous observations (Hermsmeyer, 1976; Marvin et al., 1979), we believe that the only major membrane change that appears on culturing is the high sensitivity to transmitters. It is likely that the change represents either the high inherent sensitivity of single muscle cells, which can only be measured in isolated cells, or is due to the culture procedure. The most vigorous step in the culture procedure is exposure to the dissociation enzymes; however, acute exposure of isolated blood vessels to even higher concentrations of collagenase and trypsin did not result in increased sensitivity, suggesting that removal of non-muscle cells reveals the elemental sensitivity, rather than enzyme exposure damaging the cells. Further evidence that the high sensitivity is inherent in the single vascular muscle cells is that sensitivity remained high during the entire observation period (present results; Hermsmeyer, 1976; Marvin et al., 1979).

Increased sensitivity probably comes about due to several factors, including denervation, rapid access of the agonist to more surface area of the muscle cells, elimination of agonist uptake by non-muscle cells, elimination of dilator release by non-muscle cells, and perhaps most importantly, by the process of desensitization. If our interpretation is correct, the intact vessel would be partly desensitized, even in the neonatal rat, by endogenous circulating catecholamines. There is evidence that synthesis and release of catecholamines has begun before this age (de Champlain et al., 1970), which would account for partial desensitization in the freshly isolated vessels. In the process of making cell cultures, any influence of circulating catecholamines would be removed, allowing vascular muscle cells to return to their most highly sensitive state, that which might exist before any influence of catecholamines. Under these conditions, the true inherent sensitivity of vascular muscle cells (without influences of other cells) can be determined.

This would mean that a new theory is necessary, one which recognizes the continuous suppression of transmitter sensitivity that occurs as a result of non-muscle cell influences. We therefore suggest the theory of physiological desensitization, which would imply an important role in the normal maintenance and control of blood vessel responsiveness, as well as in the ontogenetic development of the circulatory system. The theory suggests that desensitization at first severely limits the response to NE by reducing or eliminating the second dose (or exposure beyond 1 minute) response. However, after desensitization has first occurred, the cells return to a partial sensitivity but with a lesser desensitization capacity. Thus, cells continuously exposed to NE (physiological concentrations and dose frequencies) strike a balance where sensitivity is two orders of magnitude less than in non-exposed cells and desensitization can account for less than a 50% reduction in second response. The theory would imply a continuous modulation of sensitivity based on frequency and extent of stimulation that is testable in many preparations and protocols. If this theory is correct, it also suggests that there are desensitization processes which might be interruptible, either experimentally or under disease conditions, that would lead to abnormally high vascular muscle reactivity. We intend to pursue these implications of the theory, suggested by our results on isolated single cultured cells, so as to understand better the desensitization process.

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