Further evidence for the selective disruption of intercellular communication by heptanol

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Further evidence for the selective disruption of intercellular communication by heptanol. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1911–H1917, 1999.—The lack of selective gap junctional uncoupling agents has hampered evaluation of the contribution of intercellular communication to pharmacomechanical coupling and vascular contractility. Thus we further explored the utility and selectivity of heptanol as a gap junctional uncoupling agent in isolated rat aortic rings. Fifty-two aortic rings were obtained from 15 rats and were precontracted to ~75% of maximum with phenylephrine (PE). When contraction achieved steady state (~5 min), a single concentration of heptanol (200 µM) was added to each aortic ring at 1- to 3-min intervals for up to 42 min post-PE addition. At early time points (5–10 min after PE), heptanol elicited an ~50% loss of tension (i.e., relaxation). At subsequent time points post-PE, a gradual and time-dependent decrease in the magnitude of the heptanol-induced relaxation was observed until, after ~40 min, addition of heptanol was associated with little, if any, detectable relaxation. Linear regression analysis of the magnitude of the heptanol-induced relaxation vs. the square root of the elapsed time interval (from addition of PE) revealed a highly significant negative correlation (P < 0.001, R = 0.81). Studies conducted on KCl-precontracted aortic rings revealed no detectable heptanol-induced relaxation after development of the steady-state KCl-induced contraction. These data extend our previous observations to further document the potential utility of heptanol as a "relatively selective" uncoupling agent.

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tunity”) of more nonspecific agents, in particular, heptanol, has been advanced (9, 13, 15). Thus it was the explicit goal of the current study to extend the scope of previous investigations to further evaluate the potential utility of heptanol as a “relatively selective” gap junctional uncoupling agent, while simultaneously exploring the role of intercellular communication in modulating α-adrenergic contractions in vasculature. To this end, we examined the effects of heptanol on the magnitude of steady-state phenylephrine (PE)- and KCl-induced contractile responses in isolated rat aortic rings and compared those effects with the effects of heptanol during the transient, diffusion-limited phase of PE-induced contractile responses.

MATERIALS AND METHODS

Tissue preparation. A total of 52 isolated rat aortic rings were prepared from 15 Fisher-344 rats (10–12 wk old). Briefly, the aorta was denuded of endothelium by gentle rubbing of the intimal surface with a stainless steel wire as previously described (9, 13). Three to four aortic rings (~5 mm in length) were obtained from each rat. Rings were immediately placed in ice-cold Krebs-Henseleit buffer containing (in mM) 110 NaCl, 4.8 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, and 11 dextrose in double-distilled water. Aortic rings were suspended between two fishhooks and placed in jacketed isolated 20-ml organ bath chambers with fresh Krebs-Henseleit buffer. Organ bath chambers were maintained at 37 ± 0.05°C and were continuously bubbled with 95% O2 and 5% CO2 to maintain a pH 5 7.4 ± 0.01. Aortic rings were initially set between 2 and 3 g of basal tension, washed periodically with fresh buffer, and allowed to equilibrate at 37°C over a period of ~90 min. Contractions were measured isometrically with a Grass Force Displacement Transducer (model FT-03) and were recorded on a Grass Polygraph (models 7E or 7F).

Protocol design. In all experiments (52 rings from 15 animals), rat aortic rings were precontracted to 75% of maximum with either PE (200 nM) or KCl (60 mM). When the contractile response achieved steady state (~5 min after PE or KCl was added), a single concentration of heptanol [EC50 200 µM as previously reported (9, 13)] was added to each aortic ring at 1- to 3-min intervals for up to 42 min after PE was initially added.

Data analysis. Linear regression analysis of the magnitude of the heptanol-induced relaxation response vs. time elapsed after addition of PE was performed. Unless otherwise stated, all data are expressed as means ± SE.

RESULTS

Differential heptanol-induced relaxation of rat aortic rings precontracted with PE or KCl. Figure 1 shows a representative example of the effect of heptanol on both PE- and KCl-precontracted rat aortic rings. As illustrated, PE or KCl was added to the aortic ring preparation, and the contractile response allowed to achieve steady state. After achievement of the steady-state response, a single concentration of heptanol was added to the aortic ring, and the ensuing relaxation caused a loss in tension. As shown, addition of 200 µM heptanol 5–10 min post-PE was associated with a rapid, robust, and reproducible relaxation response in all aortic rings examined. In stark contrast to observations on PE-precontracted aortic rings, there was a complete absence of any detectable effect of heptanol on the magnitude of the KCl-induced contractile response in rat aortic rings. As illustrated in Fig. 1A, heptanol had little or no detectable effect on the magnitude of the KCl-induced contractile response in rat aortic rings. Importantly, this was also the case even when the measured KCl-induced tension was as little as 600 mg. The results of several similar experiments, conducted on distinct KCl-precontracted aortic rings, are summarized in Table 1. Figure 1B shows that the PE-induced contractile response is stable over the entire duration of the experimental protocol.
Time-dependent nature of heptanol-induced relaxation of PE-precontracted rat aortic rings. Figure 2 illustrates representative tracings of the effects of heptanol on the magnitude of the PE-induced contractile response 7, 15, 24, 30, and 40 min after the addition of PE. Note the gradual and time-dependent decline in the magnitude of the heptanol-induced relaxation response; until ∼40 min after the addition of PE to the aortic ring preparation, there was little or no detectable effect of heptanol on the magnitude of the PE-induced steady-state contractile response. Data collected from several experiments on distinct aortic rings were pooled for each time point and subsequently plotted as the empirically determined heptanol-induced percent relaxation vs. the total time elapsed after the addition of PE to the organ bath chamber. As shown in Fig. 3, linear regression analysis revealed a highly statistically significant negative correlation (P < 0.001; R = 0.81).

Figure 4 shows a further representative example of the completely reversible and time-dependent nature of the heptanol-induced relaxation response. As illustrated,
conductance between cell pairs and permit visualization of unitary channel events. Under such conditions, there is little question that heptanol can completely and reversibly uncouple isolated cell pairs. More recently, the use of heptanol has been extended to assess the role of intercellular communication in mediating a variety of responses in multicellular preparations (9, 11–16, 30, 38, 40). It is in this latter scenario that the mechanism of action becomes particularly critical.

In this regard, the exact mechanism of action of heptanol is still not known. However, it is thought to be related to decreased fluidity of cholesterol-rich membrane domains, resulting in a reduced open probability of the gap junction channel (1, 7, 36). Any selectivity of heptanol therefore would presumably reside in preferential intercalation at the gap junction protein/lipid interface, thus eliciting conformational changes that “gate” the gap junctional channels closed.

Without question, this highly lipophilic anesthetic agent lacks specificity at high concentrations (i.e., concentrations ≥1–2 mM). Therefore, heptanol will undoubtedly exhibit concentration-dependent actions on many aspects of cellular function that are unrelated to its gap junctional effects. Such considerations are of particular significance to the current studies, because there are many steps between receptor activation and generation of the contractile response in isolated rat aorta. A prerequisite to the utilization of heptanol as an uncoupling agent is identification of a relatively selective uncoupling action.

In fact, an algorithm for identifying reasonable experimental conditions under which heptanol may be utilized as a relatively selective uncoupling agent have been codified (9). Briefly, it was posited that heptanol concentrations that elicit ~50% relaxation in a tissue precontracted to ~75% of its maximum had a predominant, although not exclusive, action on junctional conductance. The rationale and evidence supporting this supposition have been outlined in great detail elsewhere (9, 13, 15). An explicit aim of this study, therefore, was to expand the scope of these previous investigations to further elaborate on the potential utility of heptanol as a relatively selective gap junctional uncoupling agent.

In this regard, the major new finding of this study is the observation that the ability of heptanol to elicit relaxation of PE-precontracted, but not KCl-precontracted, rat aortic rings is time dependent (Figs. 1–3) and, moreover, independent of the order of addition of PE or heptanol (Figs. 4 and 5). More specifically, immediately after achievement of a steady-state PE-induced contractile response, application of heptanol to aortic rings produces an ~50% loss of measured tension (i.e., relaxation; see Fig. 1). For all subsequent time points, the greater the elapsed time interval between addition of PE to the aortic rings and the application of heptanol, the smaller the observed relaxation response (Fig. 2). Consistent with this latter result, linear regression analysis revealed a highly significant negative correlation between this time interval and the percent relaxation of PE-precontracted aortic rings (Fig. 3). In fact, ~40 min after the addition of PE, the application of heptanol produced little or no detectable effect on the magnitude of the PE-induced steady-state contractile response. In addition, at no point in time did heptanol ever elicit a detectable relaxation response on KCl-precontracted tissues (Table 1), even when the measured tension was as little as 600 mg (see RESULTS). Finally, even when the heptanol-induced relaxation response was ~50%, within 20 min the contractile tension still returns to the preheptanol value (Fig. 4), nominally in the continued presence of both heptanol and PE. Because the heptanol was added 12 min after addition of PE in this experiment and the contractile response returned to the “preheptanol” level in ~20 min, these data are also consistent with the estimated time course of PE diffusion through the extracellular space (see below).

Before interpreting the results of these experiments, it is important to first consider the spectrum of hypothesized nonjunctional activities of heptanol. This spectrum includes, but is not limited to, the following: 1) gating effects on nonjunctional ion channels (e.g., calcium, potassium, and sodium), 2) effects on second messenger molecule formation/turnover/diffusion, 3) alterations in myofilament calcium sensitivity, 4) changes in the degree of myosin phosphorylation, etc. Most, if not all, of these processes contribute to the tonic portion of smooth muscle contraction. Thus, if heptanol had a major action on smooth muscle tone that was unrelated to its activity on gap junctions, application of heptanol should universally alter the amplitude of the PE-induced contractile response, regardless of the elapsed time from addition of PE. Certainly, this was not the case in the present experiments (see Figs. 1–4).

As elaborated elsewhere (9, 13, 15), the fact that heptanol had no detectable effect on the magnitude of the KCl-induced contractile response, even at extremely low levels of contractility, is also inconsistent with the presence of a significant nonjunctional action. That is, the KCl-induced contractile response is accompanied by lower levels of myosin phosphorylation and tension development per unit increase in intracellular calcium levels than agonist-induced contractions (i.e., α-adrenergic responses; see Ref. 26). Thus, if heptanol altered any aspect of myofilament calcium sensitization or myosin light chain phosphorylation, KCl-induced contractile responses, which are inherently less efficient with respect to second messenger formation/sensitization, should be disproportionately affected by heptanol. Such was not the case, even when the amplitude of the KCl-induced stimulus/contractile response was exceedingly low (i.e., <600 mg).

The observations of others also support the possibility of “selective” uncoupling actions for heptanol. By and large, the nonjunctional actions of heptanol on sodium, potassium, and calcium channels in cardiac and smooth muscle myocytes are observed at heptanol concentrations ≥1–2 mM (21, 32, 35, 37). The recent findings of Garcia-Dorado and co-workers (21) support the plausibility of a concentration-dependent separa-
tion of the junctional and nonjunctional actions of heptanol. Specifically, they showed that relatively selective junctional effects occurred at concentrations ranging from 30 to 300 µM, whereas nonjunctional effects occurred at heptanol concentrations from 1 to 3 mM. Other studies have suggested, but not proven, that heptanol might alter calcium channel activity in gastrointestinal smooth muscle at concentrations as low as 0.5 mM (35) or in rabbit mesenteric artery at concentrations >300 µM (9). Conversely, studies in guinea pig vas deferens have provided evidence for selective disruption of intercellular communication among smooth muscle cells at concentrations ranging from 500 µM to 2 mM (30). Such differences emphasize the importance of utilizing heptanol on a case-by-case basis, as originally proposed (9). However, taken together, the current weight of experimental evidence indicates that the heptanol concentration used here (200 µM) would be expected to elicit minimal perturbation of nonjunctional ion channels, coupled with minor effects on myocyte contractility (perhaps 5–10% as reported by Garcia-Dorado et al. [21] compared with the 50% relaxation observed at the earlier time points, for example).

If heptanol has a relatively selective action on junctional communication, then one cogent interpretation of our data is that the time-dependent effects of heptanol reflect the time course of PE diffusion through the extracellular space of the aortic media. Clearly these studies did not directly address this possibility, but the specific rationale is as follows. As PE diffuses through the medial smooth muscle layer(s) it will gradually activate all possible responsive cells (Fig. 5). When PE has directly activated all of the smooth muscle cells that are capable of responding (i.e., cells that have

Fig. 5. Schematic depiction of the putative mechanistic basis for the observed time-dependent nature of the heptanol-induced relaxation response. A: Immediately after the PE-induced contractile response achieves steady state, only a fraction of the smooth muscle cells in the vessel wall are directly activated by PE (filled smooth muscle cells), and thus the magnitude of the contractile response is quite dependent on the "indirect" activation of many smooth muscle cells via the intercellular diffusion of relevant second messenger molecules through gap junctions. Addition of heptanol disrupts this intercellular pathway, decreasing the number of smooth muscle cells that are able to participate in the contractile response and restricting them to only that fraction of cells that is directly activated by PE. B: ~20 min after addition of PE to the organ bath, a much greater fraction of the cells in the vessel wall are now directly activated by PE, and thus the observed contractile response is less dependent on intercellular communication through gap junctions. C: by 40 min after the addition of PE to the organ bath chambers, sufficient PE has diffused throughout the vessel wall to directly activate all of the cells that are capable of contributing to the PE-induced contractile response. Thus, at this point, the contractile response is virtually independent of intercellular communication through gap junctions, and there is little or no detectable effect of heptanol on the steady-state magnitude of the PE-induced contractile response. Note that this figure depicts only one possible cellular activation/extracellular diffusion profile. More specifically, it is quite clear that there are many patterns of cellular activation possible after addition of PE and that these manyfold possibilities would reflect the relative magnitude of the contribution of, for example, the degree of luminal vs. adventitial diffusion, as well as the presence of functional membrane receptors on the affected cells.
functionally coupled α-adrenergic receptors), the observed contractile response will nominally be independent of intercellular communication. As illustrated in Fig. 5C, at this point in time, the magnitude of the steady-state response would be expected to be the same whether the smooth muscle cells were directly (i.e., a cell contracts subsequent to PE activation of its α-adrenergic receptor) or indirectly [i.e., a cell is activated by the intercellular diffusion of relevant second messenger molecules/ions (e.g., calcium, inositol trisphosphate, or diacylglycerol)] activated by PE.

Despite the requisite assumptions, and numerous possible diffusion paths, it is still worth considering what the time-dependent nature of the effects of heptanol on the steady-state contractile responses to both PE and KCl might indicate about the expected extracellular diffusion profiles of these compounds, under these conditions. That is, let us assume that the homogeneous EC50 distribution of KCl or PE (i.e., one-half of the molecules) throughout the vessel wall is roughly correlated with the achievement of the steady-state contractile response. In light of this explicit assumption, one can make approximate calculations using a simple linear diffusion equation of the following form (29): \( \frac{d^2x}{dt^2} + D_{\text{app}} = 0 \), where \( x \), the diffusion distance for the one-half concentration, is 50–100 µM [assuming that there may be (to varying degrees) luminal and/or adventitial diffusion through the ~100 µM thickness of the rat aorta (13)], \( t \), is time, and \( D_{\text{app}} \) is the apparent diffusion coefficient. As such, we calculated \( D_{\text{app}} \) to be in the range of 1–3 \( \times \) 10^{-7} cm²/s for KCl (i.e., 5 min or 300 s after addition of KCl; see Fig. 1) and 2–4 \( \times \) 10^{-8} cm²/s for PE (40 min or 2,400 s after addition of PE; see Fig. 3). Clearly, the representative example illustrated in Fig. 4 further bolsters such a hypothesis and shows that the regaining of tension after the addition of heptanol to the precontracted aortic ring is also consistent with the time course for the estimated diffusion of PE through the extracellular space (i.e., ~12 min of preincubation and ~20 min to regain tension).

Finally, it is relevant to consider the potential implications of these in vitro observations to the in vivo environment. This report clearly documents that, in the presence of sufficiently high agonist concentrations (i.e., the saturating and stable PE concentrations provided in the organ bath chamber) for adequately long time intervals (~40 min), PE can directly activate all of the vascular smooth muscle cells that contribute to the contractile response. Under such conditions, the PE-induced contractile response in vitro is clearly independent of junctional communication. However, because alterations in smooth muscle tone in vivo are continuously modulated by the dynamically changing hormonal milieu, it is unlikely that either of these two conditions (i.e., saturating and stable drug concentrations) is consistently met in vivo. Thus it would seem that intercellular communication through gap junctions in vivo would play an absolutely critical role in the initiation, maintenance, and modulation of smooth muscle tone. Certainly an unequivocal affirmation of this hypothesis awaits verification in vivo.

In conclusion then, there are two important physiological implications of the current observations. First, as previously hypothesized (9, 13, 15, 16), these data provide the most compelling evidence yet that, under appropriate experimental conditions, heptanol can indeed be used as a relatively selective, as well as a readily reversible, gap junctional uncoupling agent. Second, having explicitly accepted the verity of the first supposition, it is clear that intercellular communication through gap junctions is likely to play a very prominent role in pharmacomechanical coupling and vascular contractility in vivo.

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