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Quantitation of the dose-response relationship for arrhythmogenic agents in isolated cardiac tissue

Quantificação da relação dose-resposta para agentes arritmogênicos em tecido cardíaco isolado

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Abstract
In this study, we developed an alternative approach for determination of the dose-response relationship for drugs which facilitate cardiac arrhythmogenesis by enhancing cell Ca^{2+} cycling. This approach was applied to investigate the arrhythmogenic response of isolated rat left atria to the adrenergic neurotransmitter noradrenaline (NA). The method was based on interposition of 1 min-long rest periods, during which spontaneous contractions (SC) were recorded, preceded or not by high-frequency stimulus trains (5 Hz). A given stimulation protocol was repeated at different NA concentrations ([NA]) in four preparations. The arrhythmic response to each [NA] was the sum of the number of SCs recorded at all rest periods, and data from different preparations were pooled. After normalization of the response by the number of studied atria, the relationship was fitted by a sigmoid function, for determination of Min (i.e., SC occurrence in the absence of NA), R_{max} (maximal response to NA) and EC_{50} ([NA] that evoked a response equal to 50% of R_{max}). Using, for each [NA], a single observation rest period, preceded or not by a 5 Hz train, resulted in a small arrhythmic response (R_{max} ~300 and 100 SCs/atrium, respectively) occurring only at high [NA] (≥ 10 μM). Three observation periods separated by 30 s-long stimulation periods at either 0.5 or 5 Hz resulted in considerably higher R_{max} (~600 SCs/atrium) and allowed EC_{50} determination (~5 μM). The main advantages of this method are its simplicity, the requirement of a small number of preparations and the possibility of application to other potentially arrhythmogenic agents.
Keywords: *Atrial myocardium, Catecholamines, Concentration-effect curve, Electrical stimulation, Spontaneous activity.*

Resumo
Neste estudo, descrevemos uma abordagem alternativa para determinação da relação dose-resposta para agentes que exercem efeito arritmogênico cardíaco por aumento da mobilização celular de Ca^{2+}. Esta abordagem foi aplicada na determinação da relação concentração-efeito arritmogênico para a noradrenalina (NA), que atua como neurotransmissor adrenégico, em átrio esquerdo isolado de rato. O método baseou-se na interposição de pausas estimulatórias de 1 min, durante as quais registrou-se contrações espontâneas (SCs), precedidas ou não por trens estimulatórios de alta fequência (5 Hz). Um dado protocolo estimulatório foi repetido na presença de diferentes concentrações de NA ([NA]) em quatro preparações. A resposta arrítmica a cada [NA] foi conside-

rada como a soma dos números de SCs em todas as pausas, no total de preparações. Após normalização da resposta pelo número de preparações, a relação foi ajustada por uma função sigmóide para cálculo de Min *(i.e., número de SCs na ausência de NA),* R_{max} *(resposta máxima a NA) e* EC_{50} *([NA] que produziu uma resposta igual a 50% de* R_{max}*). Quando, a cada [NA], usou-se um único período de observação, precedido ou não por um trem de alta freqüência, a resposta arrítmica foi baixa (*R_{max} *~300 e 100 SCs/átrio, respectivamente), ocorrendo apenas em alta [NA] (≥ 10 μM). Quando foram utilizados três períodos de observação separados por períodos de 30 s de estimulação elétrica a 0,5 ou 5 Hz,* R_{max} *sofreu um aumento considerável (~600 SCs/átrio) e foi possível a determinação da* EC_{50} *(~5 μM). As principais vantagens deste método são a simplicidade, a necessidade de um menor número de preparações e a possibilidade de sua aplicação a outros agentes potencialmente arritmogênicos.*
Palavras-chave: *Atividade espontânea, Catecolaminas, Curva concentração-efeito, Estimulação elétrica, Miocárdio atrial.*

Introduction

Characterization of the concentration-dependence of the pro-arrhythmic effects of chemical agents is important in both basic (e.g., studies on arrhythmia mechanisms and on susceptibility to arrhythmia in animal models of cardiovascular disease) and applied research (e.g., drug development). Estimation of an agent's efficacy (related to the response magnitude) and potency (related to the tissue sensitivity to the agent) requires determination of the concentration-effect relationship, classically described as the sigmoid function that relates the response to the logarithm of the agonist molar concentration.

However, obtaining such a relationship is not a trivial matter when arrhythmic activity is the focused response. The pattern of the arrhythmic response is often highly heterogeneous among preparations and, in a single myocardial preparation, the arrhythmic response is usually not amenable to description by a sigmoid function of the logarithm of the agonist concentration. To obtain quantitative parameters of the dose-response relationship, some investigators have considered the occurrence of arrhythmic episodes as an all-or-none phenomenon. In the latter case, the usual approach is populational, and the response is graded as the ratio of the number of preparations (each from a different animal) which develop arrhythmia and the total number of studied preparations, at each agonist concentration (e.g., Grimm *et al.*, 1998). Although allowing determination of sigmoid dose-response curves, this approach carries two major disadvantages: a) it requires a large number of preparations for each curve; and, b) it does not take into account the severity of the arrhythmic effect (since it does not discriminate between a single spontaneous contraction and a burst of spontaneous beats). Moreover, under continuous electrical pacing, it is sometimes difficult to distinguish a spontaneous contraction from one evoked by electrical stimulation.

Nevertheless, even in single preparations in which a sigmoid curve is not reliably obtained, a qualitative dose-response relationship is clear in arrhythmia generation (i.e., arrhythmia is more frequent at higher concentrations of an arrhythmogenic agent). If this agent exerts its action via occupation of specific receptors, it is reasonable to assume that the concentration-effect relationship would conform to the occupation theory, in which it is assumed that the occupation of receptors by a drug leads to a certain degree of stimulus, which, by its turn, results in a certain response level (for comprehensive reviews, see Furchgott, 1972 and Kenakin, 1984). This theory, based on the law of mass action, predicts that the fractional response of a tissue (i.e., the response as a fraction of the maximal response) is a non-linear function of the stimulus caused by a given fractional receptor occupancy. The stimulus, on the other hand, is determined by both tissue- and receptor-related factors, in addition to the free agonist concentration (*[A]*) at the receptors, so that the fractional response could be described as:

$$R/R_{max} = f\{S\} = f\{e \times [R_A] / [R_t]\} = f\{e \times [A] / ([A] + K_A)\} \quad (1)$$

where R/R_{max} is the fractional response; f is the function that relates receptor occupation and stimulus; e is the agonist efficacy; $[R_t]$ and $[R_A]$ are the total receptor concentration and the concentration of agonist-bound receptors, respectively; K_A is the equilibrium dissociation constant of the drug for the receptor.

According to this model, the response would be expected to be a hyperbolic function of the agonist concentration, or a sigmoid function of the logarithm of the agonist concentration. If this fails to happen for arrhythmogenesis in myocardial preparations, one can infer that the generation of the arrhythmic response is a complex phenomenon, which cannot be described by the receptor occupation theory, or the methodological approach used for data collection and analysis requires improvement. If the latter is the case, it is plausible to suppose that the classic concentration-effect relationship might be revealed under the appropriated methodological conditions.

Having these problems in mind, we devised a simple approach to allow quantitation of the arrhythmic re-

sponse as a function of the agonist concentration. This method was applied in the determination of the concentration-effect relationship for noradrenaline (NA) in electrically-paced left atria isolated from rat hearts. NA is a catecholamine released as neurotransmitter from adrenergic nerve terminals, and may exert its actions at the target cells through occupation of α and β-adrenoceptors (α- and β-ARs, Ahlquist, 1948).

Materials and Methods

Adult male Wistar rats (aged 10-15 weeks) were killed by exsanguination following cerebral concussion. After removal of the heart, the left atrium was dissected and mounted in a 20-ml organ bath containing modified Krebs-Henseleit solution (KS) with the following composition (mM): 115 NaCl, 4.5 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 11.1 glucose, 1 ascorbic acid, pH 7.4 at 36.5 °C by continuous bubbling with 95% O_2 / 5% CO_2. The atrium was impaled by a pair of platinum electrodes and attached by a cotton thread to an isometric force transducer (mod F-60, Narco Biosystems, Inc., Houston, Texas, USA). Contractions were recorded on paper with a polygraph (mod Narcotrace 40, Narco Biosystems, Inc.). After applying a small pre-load to the muscle, electrical stimulation at 0.5 Hz (bipolar voltage pulses, 1.2x threshold intensity, 2 ms-duration) was started (stimulator mod. SI-10, Narco Biosystems, Inc.). Approximately 20 min later, the muscle was stretched to ~90% of the optimal length, and a 40 min stabilization period was observed before NA addition started.

After stabilization of developed force, one of the following stimulation protocols was applied:

a) In its simplest version, the protocol consisted of a single, 1 min-long observation period, during which regular electrical stimulation was interrupted (group *rest*, Figure 1A);

b) in other set of preparations, the observation period was preceded by a train of 150 stimuli at 5 Hz (thus a 10-fold increase in stimulation rate), as shown in the bottom panel of Figure 1B (group *train+rest*);

c) the third variation tested consisted of three successive repetitions of the (a) protocol, i.e., three 1 min-long observation periods separated by 30 s-long stimulation periods at the regular (0.5 Hz) stimulation rate (group *triple rest*).

d) finally, the fourth protocol consisted of 3 successive repetitions of protocol (b), i.e., three 5 Hz trains, each followed by 1 min-long rest periods (group *triple train+rest*).

Four atria were used for each different stimulation protocol.

Only during rest were spontaneous contractions (SCs) computed, to avoid mistakes at distinguishing SCs from electrically-stimulated contractions. The rationale for the high frequency stimulation was to increase cell Ca^{2+} load, which commonly favors the appearance of spontaneous activity in the heart (Marbán *et al*., 1986; Bassani *et al*., 1997; Schlotthauer and Bers, 2000).

Depending on the conditions and on the preparation, isolated SCs or bursts of spontaneous beats (usually at a regular rate, see Figure 2, bottom panel), or no spontaneous activity at all, could be observed during rest. Preliminary experiments showed that stimulation at very high rates (10 Hz) lead to deterioration of the preparation.

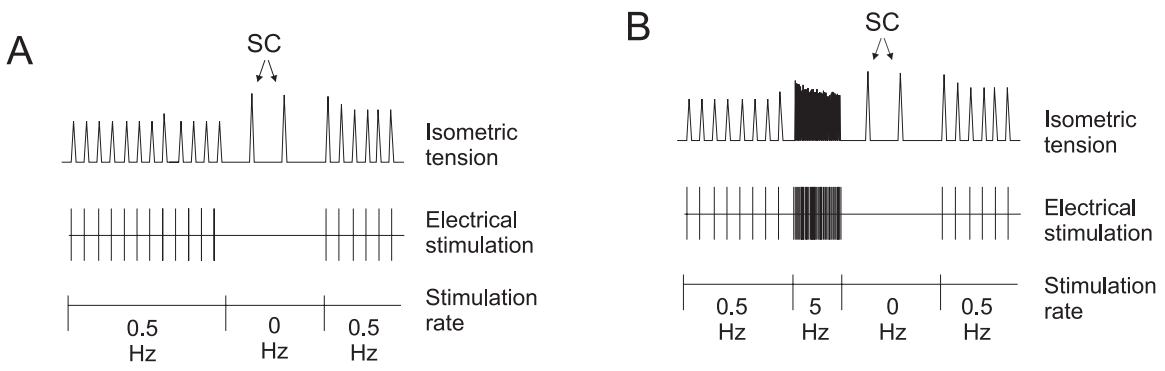

Figure 1: Diagram showing basic electrical stimulation protocols. In A, regular stimulation at 0.5 Hz is interrupted for 1 min for observation of spontaneous contractions (SC), after which stimulation was resumed (group *rest*). In B, the rest observation period is preceded by a 30 s- long train of stimuli at 5 Hz (group *train+rest*).

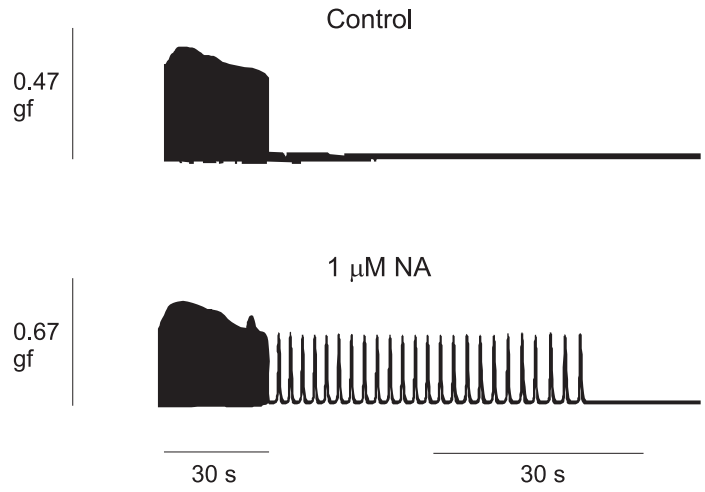

Figure 2: Original traces of force developed by isolated rat left atria showing application of the protocol *train+rest* (i.e., stimulation rate was increased from 0.5 to 5 Hz for 30 s, followed by a 1 min-long rest period), both in the absence of NA (upper panel) and after addition of 1 μM NA (lower panel). In both panels, only the 5 Hz train and the rest period are shown. Observe that, during stimulation interruption, the preparation was quiescent in the absence of NA, but developed 24 spontaneous contractions (at fairly regular intervals) in the presence of the agonist. Vertical and horizontal lines indicate force calibration signals e time. Note that time scale was expanded during the rest period.

A given protocol was applied immediately before the first NA administration, and once at each NA concentration ([NA]). After each NA addition, a 2 min period (pacing at 0.5 Hz) was allowed before the same stimulation protocol was applied. After the protocol was run, 0.5 Hz regular stimulation was resumed, and only after stabilization of the developed tension was [NA] further increased.

NA was added to the bath as to produce cumulative, logarithmic increase in [NA] in the bath solution (from 1 nM to 100 μM). Work solutions were prepared daily by serial dilution of a 20 mM NA stock solution (stored at –20 °C) in KS, and were kept on ice during use. The total amount of volume added during the experiment was < 4% of the bath volume.

For each preparation, the response was taken as the total number of SCs occurring during all the rest periods, at each [NA]. The final dose-response relationship for a given protocol was determined from the pooled data, i.e., as the sum of the number of SCs in all studied preparations, at each [NA]. The responses were then normalized, dividing the total response by the number of preparations, to express the response as average number of SC per atrium, for each [NA]. Afterward, the concentration-effect relationship was fitted by a sigmoid function (Prism 2.0, GraphPad Software, Inc., San Diego, CA, USA):

$$R = \frac{Min + (R\max - Min)}{1 + 10^{(\log EC50 - [A])}} \quad (2)$$

where [A] is logarithm of the agonist molar concentration. The parameters estimated by curve fitting were *Min*, i.e., the baseline SC rate (i.e, before NA addition); R_{max}, i.e., the highest response level (i.e., the maximal response to NA); and EC_{50}, which was the [NA] that evokes a response equal to 50% of R_{max}.

Mean EC_{50} values and the respective standard error of the mean and limits of the 95% confidence interval (CI95), determined in the curve fitting, were obtained by antilog-transformation of the values obtained by curve regression. Lack of superimposition of the CI95 was considered as an indication of statistically significant difference.

Results

In the absence of NA, the electric stimulation protocols showed to have negligible intrinsic arrhythmogenicity, in all groups. Means ± SEM of SC were zero, 0.2 ± 0.2, 0.25 ± 0.25 and 1.5 ± 1.2 SC/atrium in groups *rest*, *train+rest*, *triple rest* and *triple train+rest*, respectively (neither of these values was significantly different from zero). Observe that these are actual values, not obtained from curve fitting, such as Min values shown in Table 1.

Table 1: Parameters of the concentration-effect curves to noradrenaline (NA), determined by curve fitting, using different electrical stimulation protocols: a) a single rest observation period (group *rest*); b) a single observation period preceded by a 5-Hz train (group *train+rest*); c) three observation periods (group *triple rest*); and d) three observation periods, each preceded by a 5 Hz train (group *triple train+rest*). In all protocols, the default stimulation rate was 0.5 Hz. Mean values from curve fitting are accompanied by limits of the 95% confidence interval (in parenthesis). Four experiments were carried out in each group.

GROUP	rest	train+rest	triple rest	triple train +rest
Min[1]	-0.1	1.3	-21.0	-15.1
(SC/atrium)	(-0.2 – 0.0)	(-1.0 – 3.6)	(-76.8 – 34.7)	(-50.9 – 20.7)
R_{max}[2]	101	304	576	651
(SC/atrium)	(92 – 110)	(281 – 327)	(462 – 690)	(574 – 727)
EC_{50}[3]	163	52	4.8	5.5
(μM)	(141 – 217)	(42 – 63)	(1.9 – 12.1)	(4.1 – 9.3)

[1]Min: the number of spontaneous contractions (SC) before NA addition. [2]R_{max}: maximal arrhythmic response to NA.
[3] EC_{50}: the NA concentration that produced an arrhythmic response of 50% of R_{max}.

Although NA treatment clearly enhanced spontaneous activity (Figure 2), responses from individual atria were greatly variable. Fitted parameters in a single preparation usually presented very high standard errors (often approaching the mean value), and varied in too wide a range within the group for one to consider the means of individual values as meaningful and reliable. For instance, R_{max} ranged between 3 and 6713 SC/atrium among the individual atria in the *rest* group.

Regarding the analysis of pooled data, concentration-effect curves for the arrhythmic response to NA in the different groups are shown in Figure 3, and the curve parameters are presented in Table 1. For all the curves, the function in Eq. 2 fitted the experimental data satisfactorily ($r^2 > 0.965$, $p < 0.01$). As seen in Table 1, the mildest stimulation protocol (*rest*) produced the lowest R_{max} and the highest EC_{50} values. Introducing a high-frequency train before the observation period (*train+rest*) caused a 3-fold increase in R_{max} and a 70% decrease in EC_{50} ($p < 0.05$ for both effects). However, in both cases, most of the curve was out of the range for which it was possible to determine experimental points.

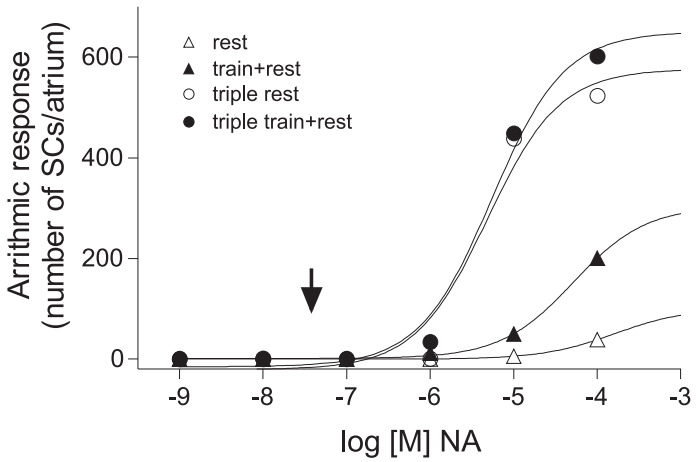

Figure 3: Concentration-effect curves to the arrhythmogenic effect of NA obtained in isolated rat left atria, using either of the following stimulation protocols at each NA concentration: 1 min-long rest observation period (*rest*), one rest period preceded by a 5 Hz stimulation train (*train+rest*), 3 rest periods, each preceded by regular stimulation at 0.5 Hz for 1 min (*triple rest*), and 3 successive applications of the 5 Hz trains followed by the rest period (*triple train+rest*). Arrhythmic response (averaged number of spontaneous contractions per atrium) is shown as a function of the logarithm of NA molar concentration. The arrow indicates the mean NA concentration which produces 50% of the maximal inotropic response in the same preparation.

Increasing the number of observation periods from 1 to 3 both increased R_{max} and decreased EC_{50} significantly. Concentration-effect curves determined with three observation periods at each agonist concentration yielded R_{max} and EC_{50} values which were not significantly different when each observation period was preceded (*triple train+rest*) or not (*triple rest*) by a high frequency stimulus train.

Discussion

In cardiac tissue, NA exerts its effects by occupation of sarcolemmal α and β-ARs, and both AR types appear to contribute to the positive inotropic response to NA and adrenaline in the mammalian myocardium (e.g., Skomedal *et al*., 1988; Jahnel *et al*., 1992). Activation of the α-AR pathway has been shown to involve, among other factors, breakdown of membrane phosphoinositides and modulation of the protein-kinase C activity. The positive inotropic effect has been associated to cytoplasmic alkalinization (via stimulation of the Na^+-H^+ exchanger), modulation of ionic currents and prolongation of the action potential (Ertl *et al*., 1991), as well as increase in the Ca^{2+} transient amplitude (Fedida *et al*., 1993; Térzic *et al*., 1993, Bers, 2001). Activation of the β-AR pathway, on the other hand, is generally accepted to involve increased synthesis of the intracellular messenger cyclic 3'-5'-adenosine-monophosphate (cAMP) and consequent activation the cAMP-dependent protein kinase (PKA). PKA, by its turn, phosphorylates several substrates, such as sarcolemmal and sarcoplasmic reticulum (SR) Ca^{2+} channels, and phospholamban, an endogenous inhibitor of the SR Ca^{2+}-ATPase, leading to an overall stimulation of cell Ca^{2+} cycling (Bers, 2001).

As a consequence of α- and β-AR stimulation, enhanced Ca^{2+} influx and/or decreased Ca^{2+} efflux, as well as enhanced SR Ca^{2+} uptake, may lead to increase in SR Ca^{2+} content and in the rate of diastolic SR Ca^{2+} release. The latter has been associated with arrhythmogenesis in mammalian myocardium, due to generation of depolarizing current in consequence of electrogenic Ca^{2+} extrusion by the Na^+-Ca^{2+} exchanger (see, e.g., Bassani *et al*., 1997; Pogwizd *et al*., 1999; Schlotthauer and Bers, 2000). Thus, it is expected that the positive inotropic effect of NA might be associated to potential arrhythmogenicity. Our present results confirm that this is indeed the case in the rat left atrial myocardium.

Accordingly, we observed that the introduction of a 5 Hz stimulation train prior to a single rest observation period caused a significant increase in both NA arrhythmogenic efficacy (as shown by R_{max} enhancement) and the atrial sensitivity to NA effects (as shown by a decrease in the EC_{50}). The latter probably occurred because enhanced response improved EC_{50} estimation. It should be stressed that 5 Hz, far from being an unphysiologically high stimulation rate, is close to the spontaneous heart rate in the rat. Thus, it appears that NA, at a physiological beating rate, is able to evoke marked spontaneous activity in the isolated atrium. Considering the single rest protocols, it is also important to remark that increasing stimulation rate, although leading to increased cell Ca^{2+} load (Bassani *et al*., 1997; Shannon *et al*., 2002), did not shown intrinsic arrhythmogenicity in the absence of NA, but rather facilitated the manifestation of NA arrhythmogenic effect. But, still, one might argue that the EC_{50} estimate would not be fully reliable with the *train+rest* protocol, because even the highest [NA] used (above which non-selective effects of the agonist might ensue) produced a response markedly lower than the estimated R_{max}, and it was impossible to ascertain whether the dose-response relationship could really be described by the classical sigmoid function.

This problem could be overcome by repeating three times the single protocols (i.e., *rest* and *train+rest*) previously tested. Preliminary observations revealed that using three separate 1 min-long rest periods resulted in greater total SC occurrence than using a single 3 min-long period, because most of SCs occur during the initial 30-40 s of the rest period (see Figure 2). This happens probably because Ca^{2+} extrusion during rest, mainly via the Na^+-Ca^{2+} exchange, leads to a progressive depletion of SR Ca^{2+} store (Bassani and Bers, 1994). On the other hand, it would not be advisable to include many observation periods because great prolongation of the exposure to the agonist might change the response to subsequent increase in [NA], due to agonist-induced receptor desensitization.

Curiously, although application of the 5 Hz stimulus train resulted in higher R_{max} during a single observation period, comparable R_{max} values were obtained with 0.5 and 5 Hz stimulation prior to rest when using the triple protocols (i.e., *triple rest* and *triple train+rest*). It is interesting to observe the distribution of SCs along the 3 successive observation periods. When atria were stimulated at 5 Hz before rest, the number of SCs was evenly distributed among the 3 rest intervals (33 ± 3%, 31 ± 4% and 36 ± 4% of the total SC number, during the first, second and third observation periods, respectively). However, when the muscle was stimulated at 0.5 Hz prior to rest, the number of SCs was very small

during the first observation period, increasing with repetition of the protocol (4 ± 2%, 38 ± 11% and 58 ± 12% of the total SC number, during the first, second and third observation periods, respectively). Thus, the use of successive observation periods seems to allow better manifestation of NA arrhythmogenic effect, not only because of sampling improvement, but also by potentiation of NA effect. It is possible that NA arrhythmogenic effect is slower to develop than the positive inotropic effect, which is stable after 2-min incubation. However, although the incubation period was the same, progressive increase in the number of SCs was not observed when rest was preceded by 5 Hz stimulation. It is likely that the initial enhancement of the Ca^{2+} load, brought about by the first 5 Hz train, might stimulate not only Ca^{2+} release from intracellular stores, which is arrhythmogenenic *per se* (Bassani *et al.*, 1997; Shannon *et al.*, 2002), but also Ca^{2+} efflux by the Na^{+}-Ca^{2+} exchange during the following rest period, so that the cell Ca^{2+} content before the second train would be lower than before the first one, and so on for further repetitions. This would antagonize the arrhythmia facilitation brought about by increase in Ca^{2+} load due to high-frequency stimulation.

We have thus shown that it is possible to obtain significant sigmoid concentration-effect curves of NA arrhythmogenic effect in isolated rat left atria, by the combination of:

a) a semi-populational approach, in which a whole curve is determined in a small set of preparations and data are pooled prior to determination of the concentration-effect curve parameters. Although working with individual curves would be preferable, the low homogeneity among atrial preparations for this type of response casts doubts on the reliability of using individual parameters to evaluate the average agonist efficacy and potency at inducing arrhythmias.
b) the use of three successive rest period for recording spontaneous activity at each [NA]. The observation that, with the use of the triple design, the arrhythmic response was not dependent on the stimulation rate prior to rest makes the experimental procedure simpler, because the whole experiment may be carried out at a single stimulation frequency. This is particularly useful because both inotropic and arrhytmogenesis responses may be determined in a single experiment. With both triple protocols, the experimental points of the whole curve could be obtained within the range of [NA] used, which makes estimation of the response parameters more reliable. The triple protocols have been routinely used in our laboratory to test NA-induced arrhythmia, and comparable R_{max} and EC_{50} values have been obtained in different pools of rat atria.

It is interesting to compare the NA EC_{50} values for positive inotropism and arrhythmogenesis. Our present estimate of the latter (~5 μM) was considerably higher than the former, previously determined in the same type of preparation (~50 nM, Bassani and Bassani, 1991, indicated by an arrow in Figure 3). As shown in Figure 3, NA arrhythmogenic effect starts to develop only at around 1 μM NA, at which concentration the inotropic response is close to its maximum. This shows a large safety margin for the effects of NA, with requirement of approximately 100-times the inotropy-stimulating effective concentration for the appearance of deleterious, arrhythmic contractions.

Conclusions

The proposed methodological approach for quantitation of the dose-effect relationship of pro-arrhythmic agents in isolated atria showed several advantages:

a) it is straightforward from the experimental point of view;
b) it requires a relatively small number of preparations, and is thus in agreement with the current worldwide policy of rationalization of the use of experimental animals;
c) although the present application has been restricted to an adrenergic agonist, the method could be used with any other chemicals which might produce dose-dependent increase in cardiac spontaneous activity by stimulation of cell Ca^{2+} cycling. Moreover, the inhibitry effect of anti-arrhythmic drugs could be also studied by analyzing their effects in decreasing efficacy and/or potency of a pro-arrhythmic agent.

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