Proc. West. Pharmacol. Soc. 38: 55-58 (1995)

Determination of the Cyclic GMP Concentration Yielding Half-Maximal Activation of Cyclic GMP-dependent Protein Kinase in Intact Ovine Basilar Arteries

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Cyclic GMP is an important second messenger in smooth muscle where it mediates a wide variety of relaxant effects, including those precipitated by nitric oxide and nitric oxide-releasing compounds such as nitroglycerin, nitroprusside and S-nitroso-N-acetyl-penacillamine [1]. Despite its regulatory importance, however, relatively little is known of the mechanisms whereby cyclic GMP produces its relaxant effects. These effects are generally attributed to the ability of cyclic GMP to activate cytosolic cyclic GMP-dependent protein kinase (G-kinase) which in turn phosphorylates a key protein or proteins within the smooth muscle cell [1]. The identity of the protein(s) phosphorylated by G-kinase remain uncertain, but have been suggested to be Ca^{2+} ATPase in either the sarcoplasmic reticulum [2] or the plasmalemma [3]. Whatever the target protein for G-kinase, its phosphorylation appears to trigger smooth muscle relaxation. This effect is terminated by dephosphorylation mediated by smooth muscle phosphatases [4].

The ability of cyclic GMP to produce relaxation varies among different arteries and different tissue types. For example, the relationship between cyclic GMP concentration and relaxation varies with age in ovine common carotid and basilar arteries [5]. Although any component of the cyclic GMP relaxation pathway mentioned above may be involved in this variation, it seems logical to first examine tissue-to-tissue variations in G-kinase characteristics. Unfortunately, most previous studies of G-kinase have employed biochemical approaches for which large amounts of tissue (> 100 g) are required [6]. In addition, these approaches require homogenization which destroys intracellular compartmentalization and thus may provide an inaccurate picture of the in vivo behavior of G-kinase. What is needed is a method to study G-kinase characteristics in small intact arteries.

In theory, it should be possible to build a mathematical model relating the time course of variation in cyclic GMP to the corresponding time course of vasorelaxation. Modern methods of cyclic GMP measurement are highly accurate, as are methods of quantitation of contractile tone. Given accurate data describing the time course of corresponding cyclic GMP and vascular tension changes, only a few reasonable assumptions should be required to estimate the concentration of cyclic GMP yielding half-maximal activation of G-kinase. The present study was conducted to explore this idea.

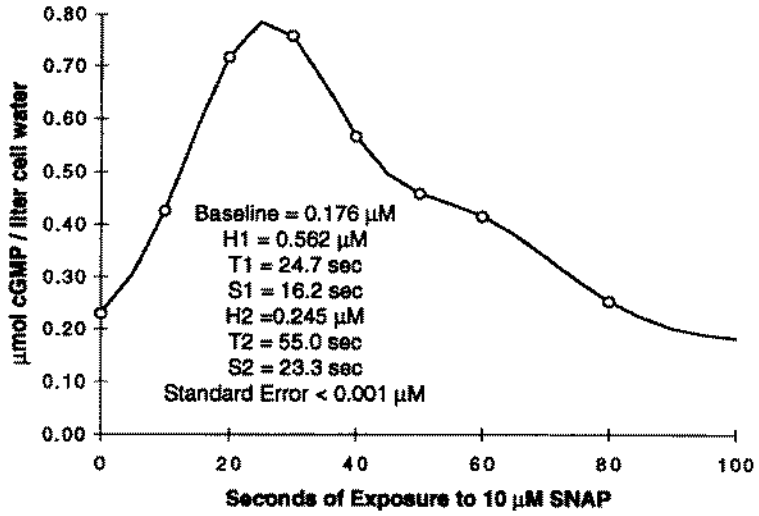
METHODS: Basilar arteries were obtained from adult male and nonpregnant female sheep, cleaned of adipose and connective tissue, and cut into approximately eight 1 mm segments. The segments were denuded of endothelium, mounted on wires and suspended in tissue baths for measurement of contractile tensions [5]. The arteries were contracted with 1 μM serotonin, then exposed for varying intervals to 10 μM S-nitroso-N-acetyl-penicillamine (a nitric oxide-releasing agent), and finally frozen. The corresponding time course of tension changes in response to S-nitroso-N-acetyl-penicillamine was measured in one segment of each artery set.

Levels of cyclic GMP were measured by radioimmunoassay and were normalized relative to cell water to estimate intracellular concentrations. Cell water was measured as the difference between total water, measured by dehydration, and extracellular water, measured as the 5^{57}Co-EDTA space, as described elsewhere [7]. To obtain a continuous series of cyclic GMP values, the cyclic GMP time course was fitted with a bimodal Gaussian equation:

$$[cGMP] = Baseline + H_1 . \exp - [(t - T_1)/S_1]^2 + H_2 . \exp - [(t - T_2)/S_2]^2$$

where H_1 is the height of the first peak, T_1 is the time to the first peak, S_1 is the spread (or SD) of the first peak, H_2 is the height of the second peak (the shoulder, in Fig. 1, Upper), T_2 is the time to the second peak, and S_2 is the spread of the second peak. The main point of using this equation was to extrapolate the input function so that cyclic GMP concentration could be known at every second of the input function. Thus, the actual form of the equation made little difference. As indicated in Figure 1, this bimodal Gaussian function fit the observed cyclic GMP time course with an average standard error of less than 0.001 μM.

The model constructed to fit the cyclic GMP time course data to the simultaneously observed relaxation responses (see Fig. 2) was based on the commonly held view [1] that cyclic GMP produces relaxation by activating cyclic GMP-dependent protein kinase (protein kinase G). In addition, it was assumed that the concentration of cyclic GMP was rate-limiting for the phosphorylation of the target protein and that the target protein was available in excess. Thus a single-order equation was written to describe the kinetics of the G-kinase phosphorylation reaction. It was further assumed that the smooth muscle phosphatase reaction whereby the target protein was dephosphorylated also followed single-order kinetics. Regarding the reaction mediating relaxation, its velocity was assumed proportional to the concentration of phosphorylated target protein. Because relaxation cannot occur in



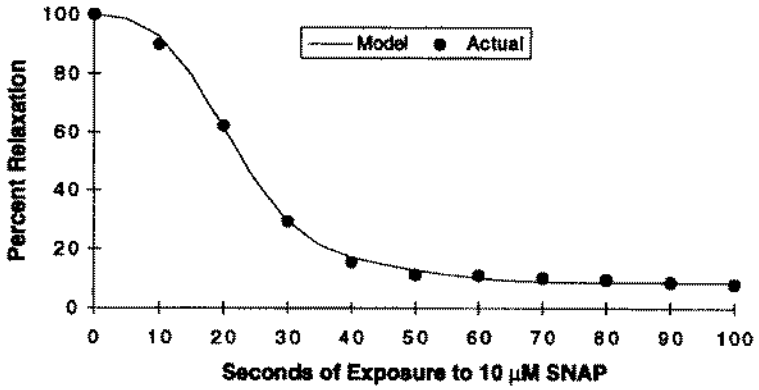


Figure 1: Time course of variations in cyclic GMP concentration and relaxation responses to 10 μM S-nitroso-N-acetyl-penacillamine (SNAP). **Upper:** Time course of cyclic GMP responses to 10 μM S-nitroso-N-acetyl-penacillamine. Each open circle represents the average of 8 animals. The solid line indicates the fit of the data by a bimodal Gaussian equation using the coefficient values indicated. Standard errors (omitted for clarity) averaged less than 0.05 μM cyclic GMP. **Lower:** The corresponding time course of relaxation produced by 10 μM S-nitroso-N-acetyl-penacillamine, measured in the same segments used for cyclic GMP analysis. Also shown is the time course of relaxation predicted by the model (see Fig. 2). Standard errors for relaxation (omitted for clarity) averaged less than 6%.

absence of initial contractile tone, this reaction was also assumed to follow second-order kinetics. Finally, because relaxation persists long after cyclic GMP has returned to baseline (see Fig. 1), the reverse rate of the relaxation reaction was assumed to be negligible. Given these assumptions and equations governing the three main reactions involved in cyclic GMP-induced relaxation, the cyclic GMP input function was fit to the simultaneously observed relaxation responses using an incremental reiterative solution strategy which minimized the mean square error between the observed and predicted relaxation responses. To constrain the model, the value of $V_{max\text{-}pkg}$ (see Fig. 2 for abbreviations) was defined as 1.0, thereby provided a reference for estimates of $V_{max\text{-}smp}$. Also for convenience, the initial percent contraction was defined as equal to 100%. For each 1 s increment, the value of [T-P] was calculated based on the amount remaining from the previous increment, and this value was used to calculate the amount of relaxation product generated per increment. This relaxation product was then subtracted from the amount of contractile tone present at the beginning of the increment to calculate the % of remaining initial contractile tone. The process was repeated 120 times (corresponding to 120 s) for each

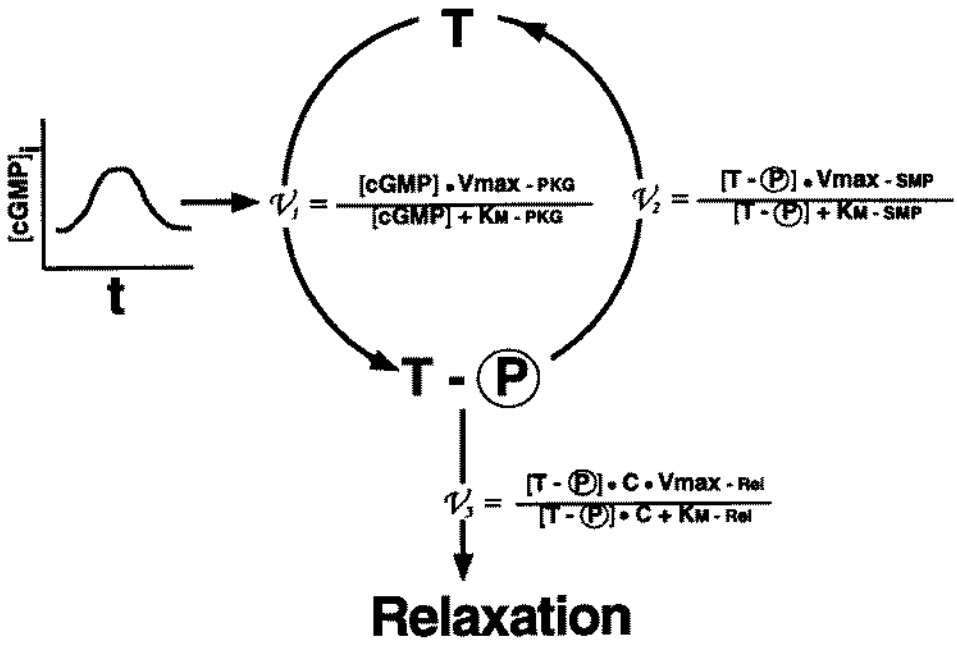


Figure 2: Shown above is the model used to fit the cyclic GMP data to the relaxation data. **Abbreviations:** **T**: target protein phosphorylated by G-kinase; **T-P**: phosphorylated form of the target protein; V_1: velocity of target protein phosphorylation; $V_{max\text{-}pkg}$: V_{max} of cyclic GMP-activated protein kinase; $K_{m\text{-}pkg}$: K_m constant of cyclic GMP-activated protein kinase; V_2: velocity of target protein dephosphorylation; $V_{max\text{-}smp}$: V_{max} of smooth muscle phosphatase; $K_{m\text{-}smp}$: K_m constant of smooth muscle phosphatase; V_3: velocity of rate-limiting reaction governing relaxation; C: contractile tone; $V_{max\text{-}rel}$: V_{max} of the rate-limiting step of the relaxation reaction; and $K_{m\text{-}rel}$: K_m constant of the rate-limiting step of the relaxation reaction.

combination of coefficient values tested. To evaluate model behavior, all coefficient sets yielding a mean square error of 5% or less were graphically analyzed by plotting error over the range of each variable.

RESULTS: The model converged on a set of coefficients which yielded an error of fit less than 2% (see Fig. 1, bottom panel). Within this set, $K_{m\text{-}pkg}$ was 0.4 μM, $K_{m\text{-}smp}$ was 4.3 μM, $V_{max\text{-}smp}$ was 8.8-fold greater than $V_{max\text{-}pkg}$, $K_{m\text{-}rel}$ was 0.49 μM% (the units here reflect the fact that $K_{m\text{-}rel}$ is a second order parameter, and is the product of 2 substrate concentrations; [T-P] in μM and an unknown component of the contractile assembly, reported as % maximum contractile tone), and $V_{max\text{-}rel}$ was 45%/s (here again, the % unit refers to % maximum initial tone). In addition, the estimate for $K_{m\text{-}pkg}$ was quite robust, as indicated by the relative insensitivity of its value to the value for the other coefficients. Within the 5% error domain, the ranges for the other variables were 10 to 1000 for $K_{m\text{-}smp}$, 2 to 2001 for $V_{max\text{-}smp}$, 1 to 10^7 for $K_{m\text{-}rel}$, and 31 to 3 X 10^{10} for $V_{max\text{-}rel}$ ($V_{max\text{-}pkg}$ being defined as one for the purposes of this model).

DISCUSSION: The relaxation time course predicted by the model on the basis of minimum error agreed well with the observed relaxation response (Fig. 1, bottom panel). The optimum value for $K_{m\text{-}pkg}$ predicted by the model (0.4 μM), agreed well with previously published results (0.29 - 0.44 μM) of studies of isolated and purified preparations of bovine aortic protein kinase G [6]. In addition, the model predicted that the value of $K_{m\text{-}smp}$ (4.3 μM) was more than an order of magnitude greater than that for protein kinase G. The model further predicted that the V_{max} value for smooth muscle phosphatase was more than 8-fold greater than the V_{max} for protein kinase G. Overall, the estimates provided by the model were realistic, which suggests that the assumptions involved were probably reasonable. If these estimates are taken to be correct, they predict that smooth muscle phosphatase has both a high K_m and a high V_{max} (relative to G-kinase), which indicates that the kinetics of the overall cyclic GMP-relaxation pathway are dominated by the properties of G-kinase.

Although the results provided by this modeling approach are indirect, they none-the-less form the basis for further studies of vascular cyclic GMP metabolism, and are of value in that they provide simultaneous estimates of

all the key coefficients involved in a single intact segment of artery. Given the robust nature of the estimates of $K_{m\text{-}pkg}$ provided by this model, it would appear to be a useful tool for studying protein kinase G when the amounts of vascular tissue available for study are small.

SUMMARY: Most previous studies of vascular G-kinase, which is widely thought to mediate the vasoactive effects of elevated cyclic GMP, have focused on the properties of the enzyme in either purified form or in arterial homogenates. How the characteristics of this enzyme might differ in intact and homogenized arteries is at present unclear. The purpose of the present study was to determine the concentration of cyclic GMP yielding half-maximal activation of G-kinase in intact arteries and to compare this estimate with corresponding values obtained from homogenized and/or purified preparations. To make this determination, ovine cerebral arteries were treated with a sub-maximal concentration of nitric oxide after which the time courses of both cyclic GMP accumulation and relaxation of serotonin-induced tone were measured. Values of cyclic GMP were normalized relative to cell water to yield apparent intracellular concentration, and this concentration time course was used as the input function for a mathematical model which related the concentration to the corresponding relaxation time course. The model assumptions were that: (i) cyclic GMP concentration was rate-limiting for G-kinase activity; (ii) smooth muscle phosphatase activity could be approximated by a single order reaction; (iii) the rate of relaxation followed second-order kinetics and was proportional to the rate of phosphorylation of the substrate for G-kinase; and (iv) the reverse rate of the relaxation reaction was very slow compared to the rate of relaxation. Using these constraints, the model converged on a K_m value for G-kinase of $\approx$ 0.4 μM, which agreed quite closely with the values reported for purified vascular G-kinase. Thus, this modeling approach appears capable of providing direct estimates of the K_m for G-kinase in small intact artery samples.

ACKNOWLEDGEMENTS: Supported by PHS grant # HL41347, American Heart Association grant# 92-293A, and by the Loma Linda University School of Medicine.

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