Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 9360–9364, December 1988
Physiological Sciences

Guanosine 5′-monophosphate modulates gating of high-conductance Ca^{2+}-activated K^+ channels in vascular smooth muscle cells

(patch clamp/vasodilation/atrial natriuretic factor/nitroprusside/signal transduction)

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Communicated by P. Roy Vagelos, July 29, 1988

ABSTRACT **Ca^{2+}-activated K^+ channels (P_{KCa} channels) account for the predominant K^+ permeability of many types of smooth muscle cells. When activated, they oppose depolarization due to Na^+ and Ca^{2+} channel activity. Several vasodilatory agents that increase intracellular cGMP levels (e.g., nitroprusside, adenosine, and atrial natriuretic factor) enhance the activity of these high-conductance P_{KCa} channels in on-cell patches of bovine aortic smooth muscle cells. In addition, dibutyryl-cGMP (1.0 mM) causes a similar increase in channel activity. To pursue the mechanism of channel modulation by these agents, a series of guanine and adenine nucleotides were evaluated by using inside-out excised patches. Whereas cAMP, AMP, ADP, and ATP were ineffective, all of the corresponding guanine nucleotides potentiated P_{KCa} channel activity when tested at a high concentration (500 μM). However, only GMP consistently enhanced channel activity in the 1–100 μM range by increasing the percent open time and frequency of opening of these channels over a wide range of potentials and Ca^{2+} levels without affecting single-channel conductance. Thus, GMP is a potent modulator of P_{KCa} channels and it, rather than cGMP, may mediate the action of the vasodilators examined in this study.**

Vascular smooth muscle is relaxed by a number of endogenous and exogenous agents that cause an increase in intracellular guanosine 3′,5′-(cyclic)-monophosphate (cGMP) (see ref. 1 for review). The nitrovasodilators (e.g., nitroprusside) and endothelium-derived relaxing factor (EDRF) activate the soluble form of guanylate cyclase (2–4), whereas atrial natriuretic factor (ANF) and adenosine act through receptors on the cell surface to activate the particulate form of this enzyme (5–8). In spite of extensive studies, the mechanism by which cGMP causes smooth muscle to relax has remained elusive. Mechanisms involving phosphorylation of critical regulatory proteins by cGMP-dependent protein kinase have been proposed, but the identity of a physiologically relevant target for this phosphorylation has not been satisfactorily demonstrated. Others (9, 10) have proposed that members of this group of vasodilators cause relaxation due to hyperpolarization of the smooth muscle cell membrane, but the process by which these agents could affect the muscle cell membrane potential has not been determined. However, in the photo- and olfactory-transduction processes in vertebrates, cGMP alters the transmembrane potential by affecting gating of a population of cation-selective channels (11, 12).

Given the abundance of Ca^{2+}-activated K^+ channels (P_{KCa} channels) in various types of smooth muscle (13), a process that increases their activity must oppose depolarizing signals, hyperpolarize the membrane, and promote relaxation. We therefore investigated the possibility that elevation of cytosolic cGMP concentration, elicited by a group of vasodilators, could modulate gating of the high-conductance P_{KCa} channels in bovine aortic smooth muscle. By the use of on-cell patch-clamp techniques, the vasodilators and dibutyryl cGMP (Bt_2cGMP) were found to potentiate P_{KCa} channel activity. Furthermore, guanine nucleotides had a similar effect when applied to the cytoplasmic surface of excised patches. However, only GMP, not cGMP, enhanced channel activity in the low micromolar range. Thus, other guanine nucleotides, besides cGMP, are involved in gating of P_{KCa} channels. Although the vasodilators cause an increase in cytosolic cGMP, other steps must be involved in mediating the enhancement of P_{KCa} channel activity. A preliminary report of these findings has appeared in abstract form (14).

MATERIALS AND METHODS

Cell Culture. Thoracic aortae of freshly slaughtered domestic calves were removed under sterile conditions. Endothelial tissue was discarded after separation by collagenase (Boehringer Mannheim) and mechanical treatment. Explants were placed in 24- or 48-well tissue culture dishes with medium [Dulbecco's modified Eagle's medium containing 10 mM Hepes, 20% fetal bovine serum, and 100 units of penicillin and 100 μg of streptomycin per ml (all from GIBCO)]. After 5 days, when the cells were confluent, they were resuspended by trypsin treatment and transferred to 6-well plates containing 4-hydroxy-D-proline (Sigma) at 100 μg/ml to inhibit fibroblast growth. This procedure was repeated, and when cells reached mid- to late-logarithmic phase, they were removed and resuspended in growth medium containing 10% dimethyl sulfoxide (American Type Culture Collection) for storage at −135°C. For patch-clamp experiments, cells were thawed and transferred to tissue culture dishes containing glass coverslips. The cells were allowed a few days to attach to the glass before they were used.

Measurements. Recordings of single-channel currents were made from cell-attached and excised patches (15). Electrodes were fire-polished (2–10 MΩ) and seals ranged from 10–50 GΩ. Experiments were performed at room temperature in a 1-ml perfusion chamber with flow rates of ≈2 ml/min. At this flow rate, absorbance measurements with dye solutions showed that ≈3 min was required for ≈95% solution exchange. Single-channel data were analyzed and histograms were generated by the PCLAMP program (Axon Instruments; Burlingame, CA). Electrode and bathing solutions are described in the text and figure legends.

RESULTS

Characteristics of High-Conductance P_{KCa} Channels in Bovine Aortic Smooth Muscle (BASM). High-conductance P_{KCa} channels in BASM cells have properties similar to those

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Abbreviations: ANF, atrial natriuretic factor; BASM, bovine aortic smooth muscle; Bt_2cAMP, dibutyryl-cAMP; E_m, membrane potential; P_{KCa} channel, Ca^{2+}-activated K^+ channel.

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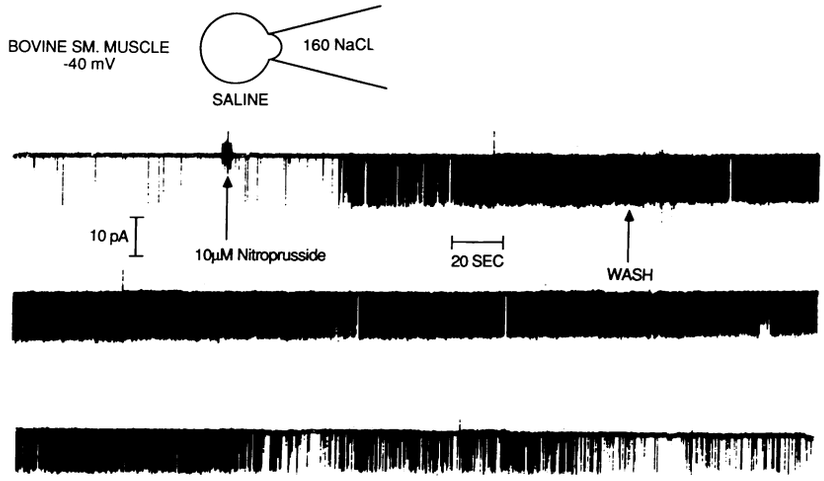

FIG. 1. Enhancement of P_{KCa} single-channel activity by nitroprusside. The microelectrode used to form an on-cell patch contained 160 mM NaCl and 10 mM Hepes (pH 7.3) and was held at a potential of −40 mV. The cell was bathed in a standard saline solution (135 mM NaCl/5 mM KCl/10 mM $CaCl_2$/2 mM $MgCl_2$/10 mM Hepes, pH 7.3). A few brief channel openings were observed in the 1.5 min preceding and the 45 sec following the beginning of perfusion with 10 μM nitroprusside. Return of channel activity to near control level required ≈10 min of washing.

described in other cell types (16). The single-channel conductance as measured in excised patches bathed on both sides with 150 mM KCl/1 mM MgCl was 266 ± 11.5 pS (n = 9). The density of channels was high, ranging from one to eight channels per patch (area ≈ 1 μm^2 of surface membrane). The Ca^{2+} sensitivity of the P_{KCa} channel (defined by percent open time vs. pCa) is negligible at pCa 5.4 and 90% open at pCa 5.0 when measured at +40 mV with bathing solutions containing 0.25 mM Mg^{2+}. Charybdotoxin, a potent and selective blocker of these channels, was found to have a K_i of ≈2.0 nM (17) and its mode of blockage is similar to that described for reconstituted P_{KCa} channel from skeletal muscle (18–20). Tetraethylammonium (Et_4N^+), a blocker of most types of K^+ channels, is most effective in blocking high-conductance P_{KCa} channels (16). Nearly complete blockage was observed in outside-out patches from these BASM cells held at a membrane potential (E_m) of −10 mV and bathed in saline containing 5 mM Et_4N^+.

Effect of Vasodilators on P_{KCa} Channel Activity in On-Cell Patches. P_{KCa} channel activity in on-cell patches was monitored before and after cells were exposed to bathing solutions containing vasodilators selected for their known action to elevate intracellular cGMP levels. Those included were nitroprusside (10 μM; n = 7), adenosine (10 μM; n = 3) and ANF (0.1 μM; n = 2). All three agents evoked an increase in the open-state probability of P_{KCa} channels. Fig. 1 shows a representative experiment in which a marked increase in P_{KCa} channel activity was elicited by 10 μM nitroprusside. In most experiments, the increase in channel activity caused by the vasodilators was so large that analysis was not performed. In one patch in which a minimal response to nitroprusside was observed, analysis of data revealed a control percent open time of 2.15%, which, 4 min after treatment, increased to 3.54%. In this case, the increase in percent open time resulted from an increase in average channel open time from 5.9 msec to 9.5 msec. However, analysis of data obtained with the three vasodilators showed that the percent open time increased due to an increase in the number of openings and their duration of opening. In general, cells were exposed to the vasodilators for a maximum of 5 min, but in other experiments cells were exposed to adenosine or nitroprusside for up to 25 min. In these cases, channel activity was noted to have a cyclical pattern, with 3- to 5-min periods of relatively normal activity between 1- to 3-min periods of intense activity.

Since the results obtained with the vasodilators were consistent with the suggestion that intracellular cGMP modulates P_{KCa} channel gating, a membrane-permeant analog of cGMP was examined. Exposure of cells to 1.0 mM Bt_2cGMP, after a lag time of a few minutes, caused a large increase in channel activity similar to that elicited by the vasodilators (Fig. 2). The lag time is in part due to the time required for solution exchange (95% in ≈3 min) and to the diffusion time required for buildup of a sufficient level of intracellular Bt_2cGMP. While these results suggest that cGMP may directly influence gating of P_{KCa} channels, they do not eliminate indirect mechanisms, such as phosphorylation of the channel by a cGMP-dependent protein kinase. Therefore, experiments using ex-

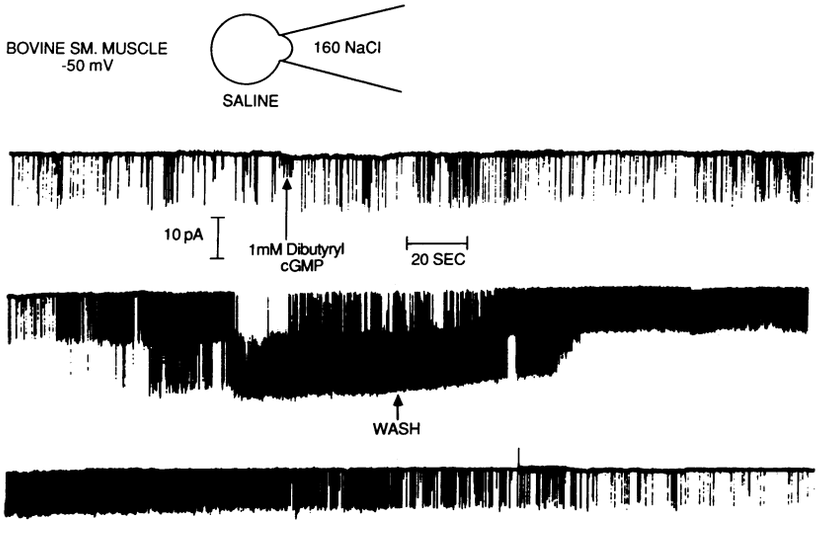

FIG. 2. Potentiation of P_{KCa} channel gating by Bt_2cGMP. The microelectrode and bathing solutions were the same as those used in the experiment of Fig. 1. In this case, however, the patch contained two P_{KCa} channels and the electrode potential was held at −50 mV. A large increase in channel activity occurred after ≈3 min of perfusion, and reversal to near control level activity was attained after 5–6 min of washing.

cised inside-out patches were performed to evaluate the possibility of channel modulation by cGMP and related agents. Two of the vasodilators, nitroprusside and adenosine, were examined on both inside-out and outside-out excised patches at the same concentrations used on intact cells, and neither agent had any effect on channel activity.

Effects of Nucleotides on P_{KCa} Channels in Excised Patches. Addition of cGMP to inside-out patches was found to potentiate channel activity, but only moderate effects were observed at concentrations below 1.0 mM. In two experiments, done under the conditions as in the experiments to be described in Fig. 3, the percent open time was measured before and after exposure of the patches to cGMP. The percent open time increased from 0.73% to 0.99% in the presence of 100 μM cGMP (E_m = +20 mV) and from 1.2% to 2.1% in the presence of 1.0 mM cGMP (E_m = +10 mV). In view of these results, GMP, GDP, GTP, and the corresponding adenine nucleotides were examined using excised patches. In one patch containing five P_{KCa} channels it was possible to test four of these nucleotides (Fig. 3). Although a high concentration (500 μM) of these agents was used, only GMP caused a frequent simultaneous opening of two to five channels. Adenine nucleotides, including cAMP, at the same concentration, had no effect (Fig. 3, bottom recording), and neither guanine nor adenine nucleotides affected P_{KCa} channel activity when applied to the external surface of outside-out patches.

The effects of GMP on P_{KCa} activity are illustrated in Fig. 4, where a sample record with expanded sections before, during, and after exposure to a buffered Ca^{2+} solution containing 50 μM GMP is shown. The arrow marked 50 μM GMP and the initial artifact on the tracing indicate the beginning of perfusion of the 50 μM GMP solution. The concentration of GMP increased slowly (see *Materials and Methods*), closely approaching 50 μM at the time the wash was initiated. In this patch, which contained only one channel, both the average open time and the number of events per unit time increased with GMP concentration. These observations are supported by data obtained from another patch whose average open time increased from 9.1 to 19.0 msec, while the number of openings increased from 254 to 1178 events per min in the presence of 100 μM GMP.

Although it seems unlikely that the necessary components required for phosphorylation of membrane proteins by a cGMP-dependent protein kinase would remain associated with excised patches, we tested for this possibility. Neither ATP (2.0 mM) nor GTP (2.0 mM), when coadministered with GMP (50 μM), caused significant potentiation over that observed with GMP alone. We also tested the possibility that a guanine nucleotide-binding protein might be involved in regulation of the P_{KCa} channel, since guanine nucleotides affect channel activity. However, neither 100 μM guanosine 5'-[γ-thio]triphosphate nor 100 μM guanosine 5'-[β,γ-imido]triphosphate (nonhydrolyzable analogs of GTP) affected channel activity in the presence or absence of GMP.

Dependence of GMP's Action on E_m and Intracellular Ca^{2+}. It is well established that the probability of P_{KCa} channel opening is dependent upon both E_m and the concentration of Ca^{2+} at the inner membrane surface. To define further the effect of GMP, the voltage dependence of channel opening was measured by using inside-out patches in the absence or presence of either 50 or 100 μM GMP. Of three patches in which open time was monitored at various E_m values, only one contained a single channel. For this single channel the probability of the open channel configuration was determined

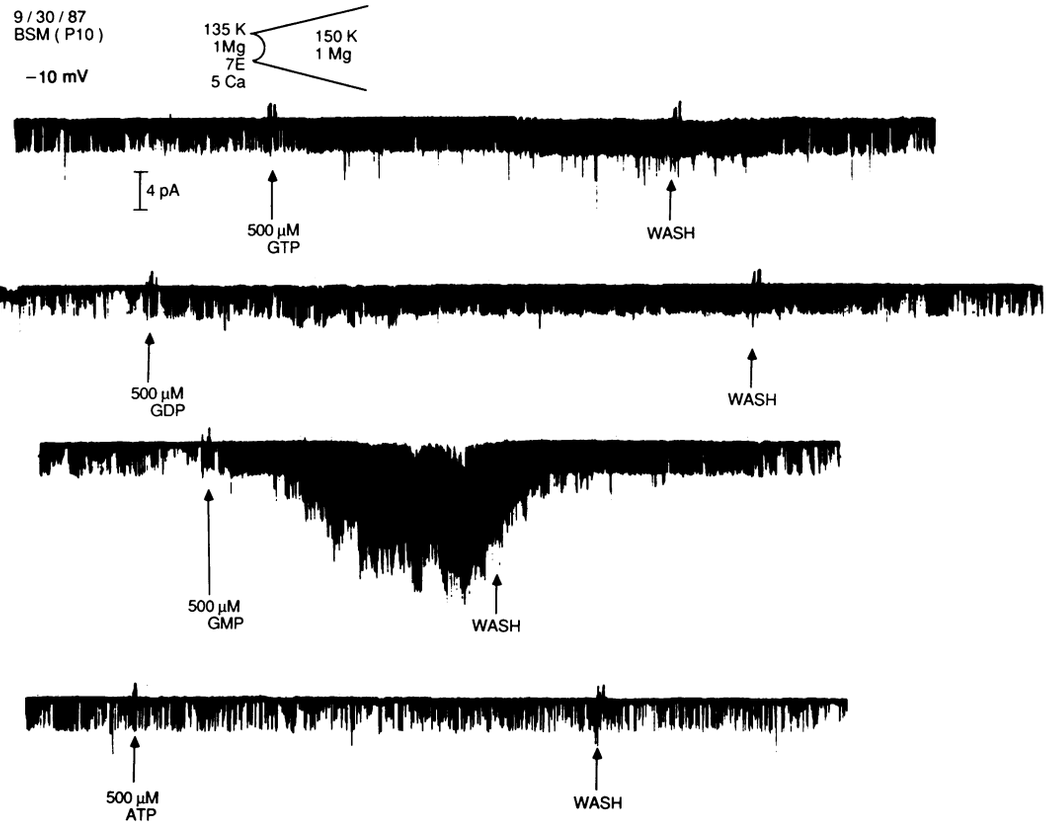

FIG. 3. Comparison of the effects of four nucleotides on P_{KCa} channel activity. For this inside-out patch, which contained five P_{KCa} channels, the electrode solution was 150 mM KCl/1 mM $MgCl_2$/10 mM Hepes, pH 7.3. A Ca^{2+}-buffered solution perfused the cytoplasmic side of the patch (135 mM KCl/1 mM $MgCl_2$/7 mM EGTA/5 mM $CaCl_2$/10 mM Hepes, pH 7.3). Ca^{2+} buffering is essential when test nucleoside tri- and diphosphates are tested, since they bind Ca^{2+} and reduce channel activity. At the high concentration (500 μM) used, GTP and GDP caused a small increase in activity. ATP had no effect and GMP caused frequent simultaneous opening of all five channels.

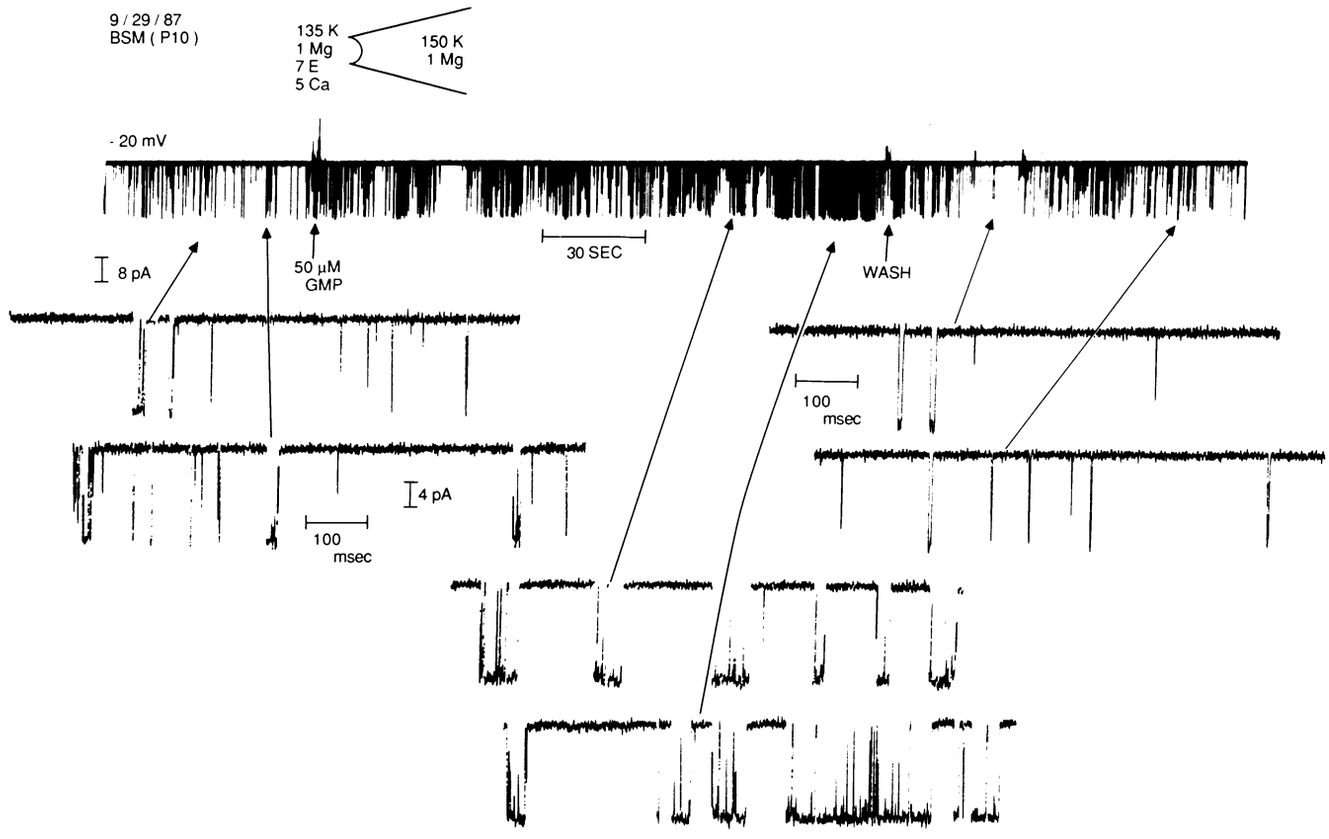

FIG. 4. GMP increases P_{KCa} channel activity. Microelectrode and bathing solutions were the same as described for Fig. 3. The inside-out patch contained one channel, and the electrode potential was −20 mV. As described in the text, the concentration of GMP increased slowly from a few micromolar at the start of perfusion of the 50 μM GMP solution to near 50 μM at the beginning of the wash. Expanded-time recordings show the increase in open time in the presence of GMP.

(Fig. 5*A*). For the other patches (containing three to five channels), average channel current was measured. In all three experiments the slope of the steepest portion of the curves was increased by GMP. This E_m dependence of GMP's modulation of P_{KCa} channels in BASM cells is similar to that observed for the interaction of cGMP with the cation-selective channels in retinal rods and olfactory receptor cells (11, 12). The enhancement of nucleotide effect with depolarization may be due to (*i*) an increase in binding of GMP to a channel conformation that is more abundant at positive membrane potentials or (*ii*) an increase in accessibility of a binding site within the voltage field.

The interaction of GMP with the channel, in addition, depends upon intracellular Ca^{2+} concentration. With two inside-out patches that contained multiple P_{KCa} channels, average channel current was measured following exposure of the patches to each of a series of buffered Ca^{2+} solutions containing 0.25 mM Mg^{2+}. With these data, plots of probability of channel opening vs. pCa were constructed for control and GMP treatment (Fig. 5*B*). Potentiation of channel activity by 100 μM GMP was marked at pCa values below about 5.5. This may have been due to an increase in GMP affinity of those channel conformational states that are more abundant in high-Ca^{2+} than in low-Ca^{2+} solutions. Although not examined in detail, the relationship between probability of channel opening and pCa was found to be highly dependent upon the concentration of Mg^{2+}. For example, with only contaminating levels of Mg^{2+} a probability of 0.5 required a Ca^{2+} concentration of several hundred micromolar.

DISCUSSION

The vasodilatory agents used in this study, as well as endothelium-derived relaxing factor, have all been shown to increase intracellular cGMP in tissues where receptors for these agents are present (2–8). It has been proposed that the vasodilatory effect arises from phosphorylation, by cGMP-dependent protein kinase, of one or more proteins that play a role in the regulation of smooth muscle contraction. For example, myosin light-chain kinase has been proposed as a phosphorylation target (21–23). In fact, myosin light-chain kinase is phosphorylated *in vitro* by cGMP-dependent protein kinase (24, 25), but phosphorylation has no effect on the activity of myosin light-chain kinase. To date, no physiologically relevant target for cGMP-dependent protein kinase has been demonstrated in smooth muscle.

The present study was undertaken to pursue the possibility that the increase in cytosolic cGMP elicited by the vasodilators modifies gating of P_{KCa} channels in a manner analogous to that described for cGMP on gating of cation-selective channels in vertebrate retinal rods and olfactory receptor cells. In the latter preparations, the conductance activated by cGMP has nearly the same selectivity for Na^+ and K^+ and this ion pathway is not regulated or activated by Ca^{2+} (11, 12). It is of comparative interest to note that the receptors for the 3′,5′-cyclic monophosphates in the retinal rods are distinct from those in olfactory cells. In the latter cells cAMP, cGMP, and cCMP activate the cation conductance with the order of potency cGMP > cAMP > cCMP. In retinal rods the receptor is specific for cGMP. In both types of cells, however, the cyclic monophosphates cause membrane depolarization and mediate sensory transduction. Since P_{KCa} channels account for a major portion of the K^+ permeability of smooth muscle, an increase in their activity would oppose excitatory inputs that cause membrane depolarization and Ca^{2+} influx through voltage-activated Ca^{2+} channels. Thus, these channels could

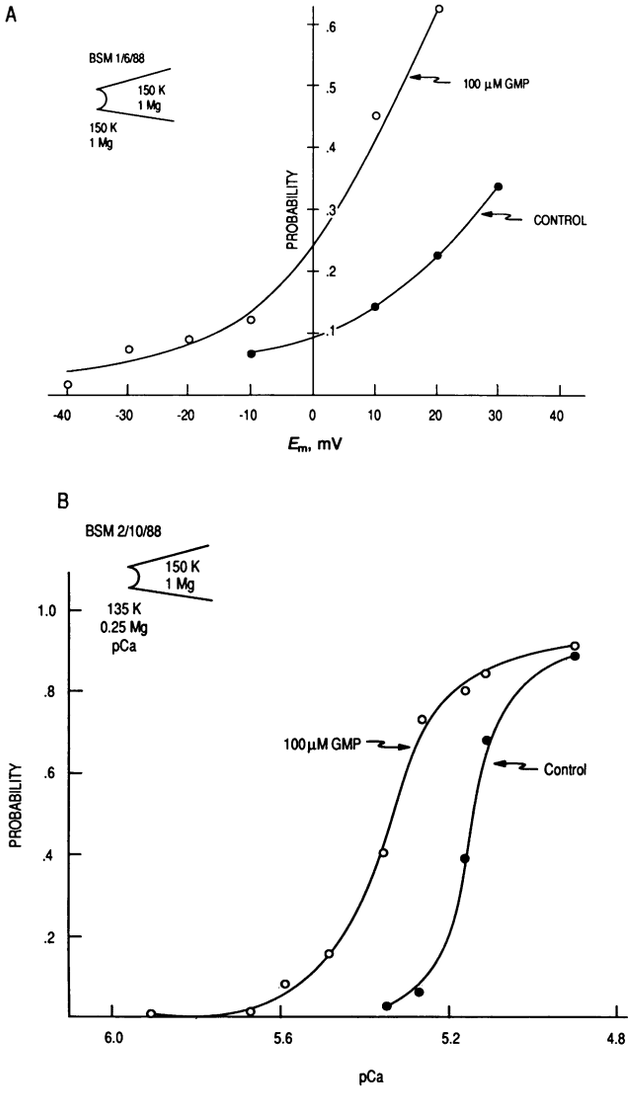

FIG. 5. (*A*) Dependence of GMP effect on E_m. In this experiment both sides of the inside-out patch were exposed to the same solution (150 mM KCl/1 mM $MgCl_2$/10 mM Hepes, pH 7.3). P_{KCa} channel activity was monitored for ≈5 min at each E_m and the patch was perfused with the 100 μM GMP solution for 5 min before data collection. (*B*) Dependence of GMP effect on Ca^{2+} concentration bathing the cytosolic side of the patch. For this inside-out patch with E_m held at +40 mV, the electrode solution was 150 mM KCl/1 mM $MgCl_2$/10 mM Hepes, pH 7.3. The bathing solution was 135 mM KCl/0.25 mM $MgCl_2$/10 mM Hepes, pH 7.0/5 mM EGTA containing various amounts of $CaCl_2$. The pCa values were calculated by using an apparent association constant of 1.92×10^6 M^{-1}.

play a major role, at least under some conditions, in smooth muscle relaxation by vasodilatory agents.

One condition under which potentiation of P_{KCa} channel activity would be expected to have a minimal effect on muscle relaxation is in the presence of high extracellular K^+, which increases membrane K^+ conductance. Under this condition, a further increase in membrane K^+ permeability (i.e., an increase in P_{KCa} channel activity) will not cause a sizable membrane hyperpolarization. Consistent with this are the findings of Winquist *et al.* (26), which demonstrate that at low extracellular K^+, the nitrovasodilators and ANF have a potent relaxing effect on vascular smooth muscle, but as extracellular K^+ is increased, the effect of these agents is diminished. Furthermore, Et_4N^+, a blocker of P_{KCa} channels, has been shown to inhibit the effect of ANF (27). While ANF could overcome the effect of Et_4N^+, 10-fold higher concentrations were required to do so.

While our results support the possibility that an increase in cGMP leads to an enhancement of P_{KCa} channel gating, it is unlikely to occur through a direct effect of cGMP on these channels. The guanine nucleotide of highest potency, and thus the most likely candidate to mediate the vasodilator's effect on P_{KCa} channels, is GMP. If GMP acts as a second messenger in a chain of events, then a phosphodiester bond must be broken after the rise in the cytosolic cGMP level. The cyclical enhancement of P_{KCa} channel activity during prolonged exposure of the vasodilators suggests that the cytosolic concentration of the final messenger might follow a similar cyclical course. This could arise due to a periodic imbalance between the production of cGMP (i.e., guanylate cyclase activity) and its degradation by a phosphodiesterase.

We thank Ms. Cindra Condra, Mr. Robert W. Babilon, Mr. John C. Rogers, and Dr. George M. Vlasuk for help with the cultured BASM cells. We are indebted to Drs. Gregory Kaczorowski and Maria Garcia, who provided invaluable insight in our many discussions.

1. Murad, F. (1986) *J. Clin. Invest.* **78,** 1–5.
2. Katsuki, S., Arnold, W., Mittal, C. & Murad, F. (1977) *J. Cyclic Nucleotide Res.* **3,** 23–25.
3. Ignarro, L. J., Lippton, H., Edwards, J. C., Baricos, W. H., Hyman, A. L., Kadowitz, P. J. & Greutter, C. A. (1981) *J. Pharmacol. Exp. Ther.* **218,** 739–749.
4. Rapoport, R. M. & Murad, F. (1983) *Circ. Res.* **52,** 352–357.
5. Waldman, S. A., Rapoport, R. M. & Murad, F. (1984) *J. Biol. Chem.* **259,** 14332–14334.
6. Winquist, R. J., Faison, E. P., Waldman, S. A., Schwartz, K., Murad, F. & Rapoport, R. M. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7661–7664.
7. Rapoport, R. M., Waldman, S. A., Schwartz, K., Winquist, R. J. & Murad, F. (1985) *Eur. J. Pharmacol.* **115,** 219–229.
8. Kurtz, A. (1987) *J. Biol. Chem.* **262,** 6296–6300.
9. Ito, Y., Suzuki, H. & Kuriyama, H. (1978) *J. Pharmacol. Exp. Ther.* **207,** 1022–1031.
10. Kreye, V. A. W. (1980) *Trends Pharmacol. Sci.* **1,** 384–388.
11. Fesenko, E. E., Kolesnikov, S. S. & Lyubarsky, A. L. (1985) *Nature (London)* **313,** 310–313.
12. Nakamura, T. & Gold, G. H. (1987) *Nature (London)* **325,** 442–444.
13. Walsh, J. V. & Singer, J. J. (1987) *Pflügers Arch.* **408,** 83–97.
14. Williams, D., Katz, G., Himmel, D. & Reuben, J. P. (1988) *Biophys. J.* **53,** 549 (abstr.).
15. Hamil, O. P., Marty, A., Nehr, E., Sakmann, B. & Sigworth, F. J. (1981) *Pflügers Arch.* **391,** 85–100.
16. Marty, A. (1983) *Trends NeuroSci.* **6,** 262–265.
17. Gimenez-Gallego, G., Navia, M. A., Reuben, J. P., Katz, G. M., Kaczorowski, G. & Garcia, M. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3329–3333.
18. Miller, C., Moczydlowski, E., Lattore, R. & Phillips, M. (1985) *Nature (London)* **313,** 316–318.
19. Anderson, C. S., MacKinnon, R., Smith, C. & Miller, C. (1988) *J. Gen. Physiol.* **91,** 317–333.
20. MacKinnon, R. & Miller, C. (1988) *J. Gen. Physiol.* **91,** 335–349.
21. Rapoport, R. M. & Murad, F. (1983) *J. Cyclic Nucleotide Protein Phosphorylation Res.* **9,** 281–296.
22. Rapoport, R. M., Draznin, M. B. & Murad, F. (1983) *Nature (London)* **306,** 174–176.
23. Rapoport, R. M., Draznin, M. B. & Murad, F. (1983) *Trans. Assoc. Am. Physicians* **96,** 19–30.
24. Nishikawa, M., de Lanerolle, P., Lincoln, T. M. & Adelstein, R. S. (1984) *J. Biol. Chem.* **259,** 8429–8436.
25. Hathaway, D. R., Konicki, M. V. & Coolican, S. A. (1985) *J. Mol. Cell. Cardiol.* **17,** 841–850.
26. Winquist, R. J., Faison, E. P. & Nutt, R. F. (1984) *Eur. J. Pharmacol.* **102,** 169–173.
27. Winquist, R., Faison, E., Napier, M., Vandlen, R., Waldman, S., Murad, F. & Rapoport, R. (1985) in *Vascular Neuroeffector Mechanisms*, eds. Bevan, J. A., Godfraind, T., Maxwell, R. A., Stoclet, J. C. & Worcel, M. (Elsevier Science, Amsterdam), pp. 349–353.