The Transduction System in the Isoproterenol Activation of the Ca\(^{2+}\)-activated K\(^+\) Channel in Guinea Pig Taenia Coli Myocytes

SHIH-FANG FAN, SHUYA WANG, and C.Y. KAO

From the Department of Pharmacology, State University of New York, Downstate Medical Center, Brooklyn, New York 11203; and Department of Physiology and Biophysics, Health Sciences Center, State University of New York at Stony Brook, Stony Brook, New York 11794

ABSTRACT In freshly dispersed guinea pig taenia coli myocytes the activity of the large conductance Ca\(^{2+}\)-activated K\(^+\) channel (maxi-K\(^+\) channel) predominates. The open probability \(P_o\) of this channel is increased by micromolar concentrations of the \(\beta\)-adrenergic agonist isoproterenol (ISO). Low concentrations of cholera toxin (CTX, 1 pM) and guanosine 5'-O-2-thiodiphosphate (GDP\(\beta\)S, 0.5 mM) suppress the ISO-induced increase of \(P_o\). Higher concentrations of CTX (e.g., 0.5 nM) as well as forskolin and dibutyryl cAMP increase the \(P_o\). 1,9-Dideoxyforskolin, the forskolin analogue, which lacks the adenylate cyclase–stimulating effect, does not. A specific protein kinase A inhibitor (Wiptide), applied intracellularly via diffusion from the patch electrode, suppresses the ISO-induced increase of whole-cell outward K\(^+\) current during step depolarization. In contrast, intracellularly applied protein kinase C (19-36), a specific protein kinase C inhibitor, has no effect on the whole-cell current. TMB-8, an inhibitor of intracellular calcium mobilization, does not affect either the whole-cell outward K\(^+\) current during step depolarization or the \(P_o\). These observations show that ISO increases the \(P_o\) of the maxi-K\(^+\) channels in the guinea pig taenia coli myocytes through the G protein–adenylate cyclase–protein kinase A system.

INTRODUCTION

Cellular systems for the transduction of extracellular stimuli into intracellular signals may involve the following components: (a) interaction of extracellular-facing receptors with stimuli, e.g., hormones, neurotransmitters, and drugs, etc.; (b) transduction through intermediate coupling proteins between the receptors and the effector molecules, a family of guanine nucleotide binding proteins (G proteins; Gilman, 1984); and (c) actions of effector molecules, which might be the ion channels (Breitwieser and Szabo, 1985; Pfaffinger, Martin, Hunter, Nathanson, and Hille, 1985) or specific enzymes, such as adenylate cyclase, which generate the second

Address correspondence to Dr. C. Y. Kao, Department of Pharmacology, Health Sciences Center at Brooklyn, State University of New York, Brooklyn, NY 11203.

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messenger cyclic 3',5'-adenosine triphosphate (cAMP) (Lefkowitz, Stadel, and Carm, 1983). In the cell membrane there is a group of receptors coupled with the adenylate cyclase-stimulating G protein (G\textsubscript{s}). Among that group is beta-adrenoceptor, a transmembrane glycoprotein mediating the action of catecholamine.

β-Adrenergic agonists increase the potassium current (I\textsubscript{K}) of cardiac myocytes (e.g., Brown and Noble, 1974; Umeno, 1984; Bennett and Begenisich, 1987; Walsh and Kass, 1988), possibly through a process involving cAMP-dependent phosphorylation (by protein kinase A) of the relevant protein (Tsien, Giles, and Greengard, 1972; Brum, Flockerzi, Hofmann, Osterrieder, and Trautwein, 1983; Kameyama, Hescheler, Hofmann, and Trautwein, 1986; Walsh and Kass, 1988). However, there are indications that I\textsubscript{K} may also be regulated by protein kinase C as well as by protein kinase A (Tohse, Kameyama, and Irisawa, 1987; Walsh and Kass, 1988).

In mammalian intestinal smooth myocytes, isoproterenol (ISO) causes hyperpolarization (Kao, Inomata, McCullough, and Yuan, 1975; Bülbbring and den Hertog, 1980) due to an increase in K\textsuperscript{+} conductance (Tomita, Tokuno, and Takai, 1985). Activation of beta-adrenoceptors is generally known to stimulate adenylate cyclase (Sutherland and Rall, 1960; Stiles, Carm, and Lefkowitz, 1984). In taenia coli myocytes of guinea pig, it also increases the intracellular cAMP concentration (Honda, Katsuki, Miyahara, and Shibata, 1970). However, it is not clear whether the increase of K\textsuperscript{+} conductance by ISO in smooth muscle cells could be mainly or exclusively accounted for by the activation of adenylate cyclase.

In cell-attached patches of smooth muscle cells of guinea pig taenia coli, there are two types of K\textsuperscript{+} channels. Under conditions where [K\textsuperscript{+}]\textsubscript{i} is 135 mM, [K\textsuperscript{+}]\textsubscript{o} is 5.4 mM, and membrane potential is ~0 mV, their conductances are 147 and 63 pS, respectively. Openings of the 63-pS channel are rare, whereas openings of the 147-pS channel are frequent. The latter are known to be regulated by both voltage and intracellular [Ca\textsuperscript{2+}] (maxi-K\textsuperscript{+} channel), and pass almost exclusively the whole-cell outward current during pulsed depolarization (Hu, Yamamoto, and Kao, 1989). In this paper we report the results of analysis of the transduction pathway of activation of maxi-K\textsuperscript{+} channels by ISO in guinea pig taenia coli myocytes.

Some preliminary results have been published (Fan, Hu, and Kao, 1989a; Fan, Wang, and Kao, 1991).

**EXPERIMENTAL METHODS**

**Materials and Solutions**

Freshly dispersed myocytes from guinea pig taenia coli were used. The method of isolation and other experimental details can be found in Yamamoto, Hu, and Kao (1989a). Single-channel events were recorded under symmetrical ion conditions; the bath and the pipette solutions used contained (mM): 140 KCl, 0.1 CaCl\textsubscript{2}, 0.6 EGTA, and 10 HEPES, pH 7.2 (adjusted with KOH) except where otherwise specified. The pCa value of this solution was 8. For recording whole-cell currents, 1 mM MgCl\textsubscript{2} and 2 mM ATP were added to the pipette solution, pH being readjusted to 7.2 with KOH. The composition of the bath solution used was the same as that used in single-channel recording. In all experiments, the bath solution was perfused at a steady rate of 1.2 ml/min.
**Electrophysiological Methods**

The conventional tight-seal method (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) was used in either the whole-cell current recording configuration or the cell-attached patch mode. The seal resistance between the patch and the pipette tip was 10–20 GΩ. For whole-cell current measurements, pCLAMP software (version 4.0 or 5.0; Axon Instruments, Inc., Foster City, CA) and the associated analog-to-digital converter and interface were used. The signals were digitized and stored directly in an IBM PC/AT compatible computer. For single-channel current measurements, the signal output from a List model EPC-7 patch clamp amplifier (Medical Systems, Corp., Greenvale, NY) was recorded on magnetic videotape via a pulse code modulation system (Toshiba model DX-900 video cassette recorder; Dagan Corp., Minneapolis, MN). The bandwidth of the amplifier was set at 10 kHz.

**Analysis of the Single Channel Current Data**

The data were played back through a 3-kHz (corner frequency) filter and an analog-to-digital converter (Neurocoder; Neuro Data Instruments Corp., New York, NY). Current distribution histograms were generated using point by point analysis. Sampling time was 100 µs. In the case of patches that showed multi-channel activities, we took the sum of the \( P_o \) of each individual open state, \( N P_o \), as the open probability of channel openings. Details of this method of analysis can be found in Brink and Fan (1989) and Ramanan and Brink (1990). The mean open and closed times were calculated according to the method of Ramanan, Fan, and Brink (1992), which can handle records with multiple channel openings.

**Drugs Used and Method of Application**

The drugs used were cholera toxin (CTX), dibutyryl cyclic adenosine 3',5'-monophosphate (dibutyryl cAMP), ISO, and propranolol (Sigma Chemical Co., St. Louis, MO); forskolin (Sigma Chemical Co. or Calbiochem-Novabiochem Corp., La Jolla, CA); 1,9-dideoxyforskolin and guanosine 5'-O-2-thiodiphosphate (GDPβS) (Calbiochem-Novabiochem Corp.); 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate (TMB-8) (Albrich Chemical Co., Milwaukee, WI); protein kinase C (19-36) (PKC1) and Walsh inhibitor peptide (Wiptide) (Peninsula Laboratories, Inc., Belmont, CA); and staurosporine (Kamiya Biomedical Co., Thousand Oaks, CA).

GDPβS, protein kinase (19-36), staurosporine, and Wiptide were incorporated into the pipette solution. With the exception of forskolin and 1,9-dideoxyforskolin, other agents were added into the bath from appropriate aqueous stock solutions. Forskolin and 1,9-dideoxyforskolin were first dissolved in dimethylsulfoxonoxide (DMSO) at a concentration of 10 mM and then diluted further with bath solution. In the experiments using forskolin and 1,9-dideoxyforskolin, concentrations of DMSO identical to that of the final drug solution (e.g., 0.5% for the experiments with 20 µM of drugs) were added in the drug-free bath solution for control recordings.

All experiments were performed at room temperature (22–24°C).

**RESULTS**

**General Description**

In the taenia coli smooth myocyte, whole-cell outward current during depolarization is carried by K⁺ (Yamamoto, Hu, and Kao, 1989b). Fig. 1A shows the effect of a bath application of 2 µM ISO on this current. Records made before and during the application of ISO to the same cell are shown. The current is increased throughout the entire duration and at all voltages. On average the peak outward current caused
by a depolarization step to +70 mV from a holding potential of −60 mV increased 92 ± 18% (mean ± SD, n = 5). The increase usually disappeared within 2–3 min after ISO was washed out. In 40% of all myocytes studied, $I_K$ declined slightly over a period of 30–40 min, even when untreated with any agent. Thus, a complete or near complete recovery shows that the myocyte was in a good state. The effect was completely suppressed by a beta-adrenoceptor antagonist, propranolol (2–10 μM; results not shown).

As reported by Hu et al. (1989), the outward current in the taenia coli myocyte is
due almost exclusively to openings of maxi-K⁺ channels. Fig. 1B shows the effect of ISO on the activity of such channels in a cell-attached patch. In this particular patch, multiple open states could be seen, especially at a highly positive (70 mV) holding potential, suggesting the presence of several active channels. At holding potentials of 55 and 70 mV, the $N_P$'s were 0.012 and 0.12 before the application of ISO and 0.15 and 0.48 during (10 min) bath application of 2 μM ISO. On average the $N_P$ at 70 mV increased 205 ± 48% (n = 6). The increase in $N_P$ is mainly due to the shortening of the average closed time of the channel. For the experiment shown in Fig. 1B with a holding potential of +55 mV, the average open and closed times are 5.9 and 405 ms before the application of ISO and 9.7 and 67 ms during the application. The average values of open time before and during the application of ISO from seven experiments were 6.2 ± 1.6 and 8.8 ± 2.2 ms, respectively, with $P =$ 0.05 for the difference from Student's $t$ test. In all the patches studied (n = 26), the unitary conductance of the channel was not affected by 2–5 μM ISO (changes were < 3% with $P > 0.1$).

Similar to observations in the whole-cell configuration, the increase of $N_P$ by ISO was suppressed completely by prior application of propranolol (2–10 μM, $n = 8$; results not shown).

**Effect of Agents Affecting Pathways Other Than That via Protein Kinase A**

Gₛ, being activated by ISO, might activate phospholipase C (cf. Martis, 1991), which in turn, via its substrate products, 1,2-diacylglycerol and inositol-1,4,5-triphosphate,
Figure 3. TMB-8, an inhibitor of intracellular Ca\(^{2+}\) mobilization, does not interfere with ISO action on the Ca\(^{2+}\)-activated K\(^+\) current. (A) Whole-cell current. Holding potential -60 mV; depolarizing steps to -20, -5, 10, 25, 40, 55, 70, and 85 mV. Pretreatment of the myocyte with TMB-8 for 10 min did not prevent the subsequent effect of ISO in increasing the outward current. (B) Single-channel activities. Cell-attached patch. Holding potential 45 mV. For each condition, a sample recording and the current distribution histogram are shown. Most of the
activates protein kinase C and mobilizes Ca\(^{2+}\) from the intracellular calcium pool. The possible involvement of those two pathways was tested first.

*Staurosporine and protein kinase C (19-36).* The effect of the protein kinase C inhibitor staurosporine (Tamaoki, Nomoto, Takahashi, Kato, Morimoto, and Tomita, 1986; Watson, McNally, Shipman, and Godfrey, 1988) was first tested. Staurosporine inhibits protein kinase C with \(k_i\) of 0.7 nM. In two of four cells 1 \(\mu\)M staurosporine (bath application) decreased the ISO-induced increase of \(N_{Po}\) of the maxi-K\(^+\) channel (the increase was <20% of that without staurosporine; data not shown). However, the specificity of staurosporine for protein kinase C is relatively low, as it also inhibits the activity of other protein kinases, including protein kinase A (\(k_i = 7\) nM). For this reason, another specific protein kinase C inhibitor, protein kinase C (19-36), was tested.

The regulatory domain of protein kinase C contains an amino acid sequence between residues 19 and 36 that resembles a substrate phosphorylation site. A synthetic peptide corresponding to this domain, PKCI acts as a potent specific antagonist with a \(K_i\) of 147 nM (House and Kemp, 1987). Intracellular application of 10 \(\mu\)M PKCI through the pipette solution has no effect on the ISO-induced increase of outward current during step depolarization (n = 5; Fig. 2 is one example). In these experiments, 2 \(\mu\)M ISO increased the peak outward current during a step depolarization to 75 mV from a holding potential of -60 mV by 87 ± 21%. The difference between this value and that caused by ISO alone, i.e., 92 ± 18%, is statistically insignificant (\(P > 0.1\)). In hippocampal neurons where protein kinase C influences the calcium current, 1 \(\mu\)M PKCI in the patch pipette is sufficient to inhibit the protein kinase C activity (Keyser and Alger, 1990). In this study, PKCI alone has no effect at either 1 or 10 \(\mu\)M. Therefore, the results strongly suggest that protein kinase C is not essentially involved in the ISO-induced increase of the outward K\(^+\) current.

*Inhibitor of intracellular calcium mobilization: TMB-8.* TMB-8 is an inhibitor of calcium mobilization from intracellular calcium stores (Chiu and Malagodi, 1976; Hunt, Silinsky, Hirsh, Ahn, and Solsona, 1990). It has been shown that in vascular smooth muscle with plasma membrane permeabilized with detergent, bath application of TMB-8 exepts its full effect within 1 min (Ishihara and Karaki, 1991). Bath application of 50 \(\mu\)M TMB-8 did not suppress the ISO-induced increase of the whole-cell outward current during step depolarization (n = 6; Fig. 3A is one example). In these experiments, 2 \(\mu\)M ISO increased the peak outward current by a 70-mV depolarizing pulse from a holding potential of -60 mV by 94 ± 12%. Again
Figure 4. Inhibitory effects of a low concentration (1 pM) of CTX. (A) Whole-cell recording. Holding potential -60 mV; depolarizing steps to -10, 10, 30, 50, 70, 90, and 110 mV. Outward current was increased by ISO (2 μM, 10 min). Further addition of CTX (1 pM) promptly reduced the larger outward current, the effect being manifested within 2 min after

A
Control

2 μM Isoproterenol

2 μM Iso + 1 pM CTX

B
Control

1 pM CTX

1 pM CTX + 2 μM Iso

C
Control

1 pM CTX

1 pM CTX + 2 μM Iso
there is no statistically significant difference between this value and that obtained with ISO alone. To obviate any problem of permeation through the unpermeabilized plasma membrane, TMB-8 was also applied directly intracellularly by diffusion from the pipette solution. Again, TMB-8 (50 μM) had no effect on the ISO increase of outward current during step depolarization (n = 5).

Bath application of 50 μM TMB-8 also had no effect on the NPo of the maxi-K+ channel that had already increased by ISO (n = 4; Fig. 3 B). The myocyte was first treated with ISO alone, whereupon NPo increased by 66%. 10 min after further addition of TMB-8 to the bath, there was no sign of any decrease of the NPo. The effect of ISO usually vanishes within 5 min after washout of the drug. If TMB-8 had any suppressive effect on ISO-induced increase of NPo, it should be manifested after a few minutes. Thus, TMB-8 has no apparent suppressive effect on the ISO effect.

Effects of Agents Affecting the Protein Kinase A Pathway

The possible pathway of activation of protein kinase A by ISO is: beta-adrenoceptor → Gs → adenylate cyclase → cAMP → protein kinase A. The effect of relevant inhibitors or activators was tested individually.

Cholera toxin (CTX) is known to have dual actions. On the one hand, by selectively catalyzing the ADP ribosylation of Gs, it inhibits the hydrolysis of GTP associated with Gs and thereby increases the activity of adenylate cyclase (Cassel and Selinger, 1977; Gill and Meren, 1978; Moss and Vaughan, 1988). On the other hand, it also decreases the efficacy of ligand activation of Gs-coupled receptors (Stadel and Lefkowitz, 1981; Cote, Grewe, and Kebabian, 1982). The minimum concentration that causes a significant increase in cAMP level in cells is 10 pM (Spiegel, 1990), whereas that affecting the ligand activation of Gs-coupled receptors is an order of magnitude lower (picomolar; Shen and Crain, 1990). Therefore, the effect of 1 pM CTX was tested first, followed in turn by the effects of GDPβS, of higher concentrations of CTX and forskolin, of dibutyryl cAMP, and of the specific kinase A inhibitor, Wiptide (Scott, Fischer, Takio, Demaille, and Krebs, 1985; Cheng, Kemp, Pearson, Smith, Misconi, van Patten, and Walsh, 1986).

1 pM CTX. This low concentration of CTX was tested on three whole-cell recordings and three cell-attached patch recordings. Whereas bath application of CTX alone at this concentration had no effect, when used with ISO it suppressed both the increase of outward K+ current during step depolarization and the increase of the NPo of the K+ channels. In whole-cell recordings, CTX either decreased the amplitude of the outward current that had already been increased by ISO (Fig. 4 A),
FIGURE 5

A

Control

2 μM Isoproterenol

2' in 2 μM Iso + 50 pM CTX

8' in 2 μM Iso + 50 pM CTX

B

Washout

0.5 nM CTX
or, if applied beforehand, prevented the increase of outward current by ISO (Fig. 4 B). Fig. 4 C shows that on the cell-attached patch, 1 pM GTX prevented the ISO-induced increase of the $N_P_o$ of the maxi-K$^+$ channel.

**GDP**β**S**. The catecholamine activation of adenylate cyclase depends on the successful binding of GTP to the regulatory guanine nucleotide site of $G_o$. The hydrolysis of the bound GTP to GDP terminates the activation. The GTPase reaction generates a tightly bound GDP at the regulatory site. Beta-adrenoceptor, after binding with its agonist, facilitates the displacement of the bound GDP by GTP. The GDP analogue, GDPβS, has a high affinity for the regulatory guanine nucleotide and is resistant to hydrolysis or phosphorylation by the nucleotide triphosphate regeneration system (Eckstein, Cassel, Lefkovitz, Lowe, and Slinger, 1979). The stimulatory effect of catecholamine on adenylate cyclase is thus nullified. When 0.5 mM GDPβS was applied intracellularly through the pipette solution, 2 µM ISO could no longer increase the whole-cell outward current during step depolarization ($n = 3$). Because GDPβS can affect $G$ proteins other than $G_o$, we only take these results as not contradicting a view that $G_o$ is involved in the transduction pathway.

**0.5 nM CTX.** The higher concentration of CTX activates adenylate cyclase. Bath application of 0.5 nM CTX increased the whole-cell outward current induced by step depolarization ($n = 4$) as well as the $N_P_o$ of the maxi-K$^+$ channel ($n = 3$). Fig. 5 A shows a typical result of the increase of the $N_P_o$. As the $N_P_o$ of the channel was already increased by CTX, addition of ISO did not cause further increase. It is worth noting that in three of five experiments, when 50 pM CTX was added after the outward current was increased by ISO, the outward current was first decreased and then increased beyond that before treatment (Fig. 5 B). This sequential effect may be explained by assuming that at 50 pM, CTX lowered the efficacy of ligand activation of $G_o$ faster than it enhanced the activity of adenylate cyclase.

**Forskolin and 1,9-dideoxyforskolin.** Bath application of 20 µM forskolin, which can activate adenylate cyclase, increased both the outward current during step depolarization ($n = 4$) and the $N_P_o$ of the maxi-K$^+$ channel ($n = 4$). 1,9-Dideoxyforskolin is an analogue of forskolin, which does not activate adenylate cyclase and is often used as a negative control to show that the forskolin effect is exerted via activation of adenylate cyclase (Joost, Habberfield, Simpson, Laurenza, and Seamon, 1988; Laurenza, Sutkowski, and Seamon, 1989; Schmidt and Kukovetz, 1989). Bath application of 20 µM forskolin Ca$^{2+}$-activated Ca$^{2+}$-dependent protein kinase. The initial phase of the increase of the $N_P_o$ may be due to a direct effect of CTX on the channel. The later phase may be due to an effect of GDPβS on $G_o$ to allow a more efficient activation of adenylate cyclase by the GTP constitutively bound to $G_o$. The stimulatory effect of the GDP analogue (e.g., GDPβS) on adenylate cyclase is thus nullified. When 0.5 mM GDPβS was applied intracellularly through the pipette solution, 2 µM ISO could no longer increase the whole-cell outward current during step depolarization ($n = 3$). Because GDPβS can affect $G$ proteins other than $G_o$, we only take these results as not contradicting a view that $G_o$ is involved in the transduction pathway.

**Figure 5 (opposite).** Effects of high concentrations of CTX. (A) Stimulatory effects of 0.5 nM CTX. Cell-attached patch. Holding potential +35 mV. (Top) Sample record and current distribution histogram before application of CTX. Unitary current peaked at 8.1 pA. $N_P_o = 0.11$. (Middle) 10 min after addition of CTX (0.5 nM) to bath medium. Channel openings are markedly increased, not only at 8.1 pA but also at 16.2 pA (two-channel openings). $N_P_o = 0.35$. In the face of such high channel activity, addition of ISO (2 µM) did not further increase openings (not shown). (Bottom) 10 min after washout of CTX and ISO. The channel activities are almost back to the control level. (B) Dual inhibitory and stimulatory effects of 50 pM CTX. Whole-cell currents. Holding potential −60 mV; depolarizing voltage steps to 0, 30, 60, 90, 120, and 150 mV. ISO (2 µM) increased outward current. Addition of CTX (50 pM) caused an initial (at 2 min) decline in the ISO-enhanced outward current and a later (at 8 min) increase of the ISO-enhanced outward current.
application of 20 μM 1,9-dideoxyforskolin did not affect either the whole-cell outward current during step depolarization or the $N_P_o$ of the maxi-K$^+$ channel. Fig. 6 shows experimental results obtained from one cell-attached patch. After taking three consecutive control records at intervals of 5 min, the myocyte was treated with
Figure 7. Effect of dibutyryl cAMP on the activities of the maxi-K⁺ channel. Cell-attached patch holding potential +65 mV. (Left) Control condition. Channels are already active because of the positive holding potential. Channel activity, with dibutyryl cAMP, dibutyryl cAMP (2 μM), was added to the bath solution. The record taken at 5 min already shows a marked increase in channel activity, with active multiple channel openings. NPₐ = 0.60. (Right) 20 min after washout. Channel activity has returned to control level.
20 μM 1,9-dideoxyforskolin for 10 min. The \( N_{Po} \) of the channel was unchanged. The agent was then washed out. 20 min later, 20 μM forskolin was introduced. The \( N_{Po} \) increased nearly 10 times (from 2.4 to 23.1%). In two experiments we used 100 μM forskolin; after the \( N_{Po} \) has increased to a steady value, addition of 2 μM ISO did not cause any further increase of \( N_{Po} \). In six other experiments, the myocytes were first treated with 2 μM forskolin. After forskolin was washed out, the myocytes were treated with 100 nM ISO. Finally, after ISO was washed out, the myocytes were treated with a combination of 2 μM forskolin and 100 nM ISO. In three of these experiments the increase in \( N_{Po} \)'s under the influence of the two agents combined was greater than the sum with either agent alone. They were ~1.3, 1.4, and 1.6 times that of the sum, respectively. In the other three experiments, the \( N_{Po} \)'s in the combination were about the same (0.92, 0.93, and 1.02 times the sum with each agent alone, respectively).

**Dibutyryl cAMP.** Bath application of a membrane-permeable cAMP analogue, dibutyryl cAMP (2 mM), appreciably increased the \( N_{Po} \) of maxi-K+ channel (\( n = 4 \)). Fig. 7 shows a typical example. In this patch, held at 65 mV, the \( N_{Po} \) of the channels increased from 0.04 to 0.60 after 5 min in 2 mM dibutyryl cAMP.

**Walsh inhibitor peptide (Wiptide).** Wiptide is a synthetic, potent, competitive inhibitor of protein kinase A with a \( K_i \) of 2.3 nM (Cheng et al., 1986). Its specificity for the cAMP-dependent protein kinase A is shown by its ineffectiveness against even such a closely homologous enzyme as the cGMP-dependent protein kinase (Glass, Chen, Kemp, and Walsh, 1989). 10 μM Wiptide applied intracellularly completely suppressed the ISO-induced increase of outward current during step depolarization (\( n = 3 \); Fig. 8). The ISO effect usually becomes appreciable within 1–2 min. In
myocytes treated with intracellular Wiptide, the outward current did not increase even after ISO had been applied for 10 min. ISO was added 20 min after making the patch. In the experimental results shown in Fig. 2, 2 μM ISO added 20 min after making the patch increased the outward current. Thus, it is clear that 30 min (20 min waiting time for Wiptide to diffuse into the cell and 10 min with ISO added) after making the whole-cell patch, ISO should still be capable of increasing the outward currents if the increase has not been suppressed by Wiptide. This point has been verified in five other experiments.

**Discussion**

Receptor agonists can modulate the activity of ionic channels by different mechanisms: (a) direct action on the receptor-ionophore complex, e.g., the 5-HT₃ receptor channel (Derkach, Surprenant, and North, 1989; Yakel, Shao, and Jackson, 1990); (b) activation of the G protein via the receptor, which in turn stimulates the ionic channel through a membrane-delimited, direct pathway, e.g., adrenergic receptors coupled to the calcium channel (Yatani and Brown, 1989) or the voltage-dependent sodium channel (Schubert, VanDongen, Kirsch, and Brown, 1989) in cardiac myocytes, and (c) modulation by Gₛ protein of the channel activity indirectly through diffusible cytoplasmic second messengers. In cardiac myocytes, adrenergic receptors are also coupled to calcium channels (Yatani and Brown, 1989) and to voltage-dependent sodium channels (Schubert et al., 1989) via the Gₛ by indirect cytoplasmic pathways. In our experiments on cell-attached patches of taenia coli myocytes, ISO and other agents were applied to the extra-patch bath solution. The gigaohm seal between the patch pipette and the cell membrane forms a barrier against the lateral diffusion of these agents onto the patch or into the pipette. Thus their effects on the channel under observation can only be attributed to actions of diffusible intracellular second messengers.

The following results of our experiments indicate that cAMP is the second messenger: (a) agents that can block the production of cAMP via the activation of beta-adrenoceptor (propranolol and low concentrations of CTX) suppress the ISO increase of the channel activity; (b) agents that can activate adenylate cyclase (higher concentrations of CTX and forskolin) as well as the membrane-permeable cAMP analogue, dibutyryl cAMP, directly increase the channel activity; and (c) forskolin at low concentrations has a synergistic effect with low concentrations of ISO, while high concentrations of either forskolin or CTX mask the effect of ISO. These results indicate that ISO and forskolin as well as CTX have a common pathway of action. Presumably, with high concentrations of forskolin or CTX the capability of the pathway is saturated and no further increase is possible.

It is well known that besides the activation of adenylate cyclase, forskolin has cAMP-independent effects (Laurenza et al., 1989). However, the following results show that the forskolin-induced increase of maxi-K⁺ channel activity in our case is cAMP dependent: (a) the ability of high concentrations of CTX and dibutyryl cAMP to produce the same effect and (b) the inability of 1,9-dideoxyforskolin to produce the same effect (cf. Laurenza et al., 1989).

In our whole-cell experiments, bath-applied ISO can act directly on any existing receptor-ionophore complex or on receptors near the channel that might act on
channels through some membrane-delimited pathway. However, the following results indicate that without the intervention of cAMP as the second messenger system, ISO only affects the channel activity negligibly, if at all. Blocking the production of cAMP via beta-adrenoceptor by low concentrations of CTX and GDPβS, as well as inhibiting the activity of the cAMP-dependent protein kinase, protein kinase A, by Wiptide completely suppress the effect. We tested the effect of ISO on three outside-out excised patches. No increase of the activity of maxi-K⁺ was observed. This observation also supports the conclusion that ISO does not increase the channel activity by acting directly on any existing receptor-ionophore complex, or by Gs through a membrane-delimited, direct pathway.

It has been shown that phospholipase C can be activated by pertussis toxin-insensitive G proteins. Those G proteins are heterotrimeric rather than small molecular weight G protein (cf. review by Martis, 1991). To our knowledge, in taenia coli myocytes those G proteins have not been identified. The activity of the maxi-K⁺ channel is highly sensitive to intracellular Ca²⁺, and might also be modulated by protein kinase C (Tohse et al., 1987; Walsh and Kass, 1988). The possibility that activation of Gs might also activate phospholipase C, leading in turn to sequential activation of inositol-1,4,5-triphosphate and protein kinase C, and mobilization of Ca²⁺ from the intracellular calcium pool, is worth verifying. That neither the protein kinase C inhibitor, protein kinase C (16-39), nor the intracellular Ca²⁺ mobilization inhibitor, TMB-8, suppresses the ISO-induced increase indicates that these pathways are less likely to be involved than that of protein kinase A.

That the activity of maxi-K⁺ channel is regulated by cAMP-dependent protein kinase has been demonstrated in various excitable cells, including smooth muscle cells. For instance, in aortic smooth myocytes (Sadoshima, Akaike, Kanaide, and Nakamura, 1988) and airway smooth myocytes (Kume, Takai, Tokuno, and Tomita, 1989; Savaria, Lanoue, Cadieux, and Rousseau, 1992) the Po of maxi-K⁺ channels is increased by protein kinase A phosphorylation.

In summary, although we do not yet know if the channel protein itself is the substrate of protein kinase A, our results show that in guinea pig taenia coli myocytes, activation of the beta-adrenoceptor enhances the activity of the maxi-K⁺ channel via the receptor-Gs-adenylate cyclase-cAMP-protein kinase A pathway.

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