Pharmacology and modulation of $K_{\text{ATP}}$ channels by protein kinase C and phosphatases in gallbladder smooth muscle

T. A. Firth, G. M. Mawe, and M. T. Nelson

Departments of Anatomy and Neurobiology and Pharmacology, College of Medicine, University of Vermont, Burlington, Vermont 05405

Firth, T. A., G. M. Mawe, and M. T. Nelson. Pharmacology and modulation of $K_{\text{ATP}}$ channels by protein kinase C and phosphatases in gallbladder smooth muscle. Am J Physiol Cell Physiol 278: C1031–C1037, 2000.—ATP-sensitive $K^+$ ($K_{\text{ATP}}$) channels exhibit pharmacological diversity, which is critical for the development of novel therapeutic agents. We have characterized $K_{\text{ATP}}$ channels in gallbladder smooth muscle to determine how their pharmacological properties compare to $K_{\text{ATP}}$ channels in other types of smooth muscle. $K_{\text{ATP}}$ currents were measured in myocytes isolated from gallbladder and mesenteric artery. The potencies of pinacidil, diazoxide, and glibenclamide were similar in gallbladder and vascular smooth muscle, suggesting that the regions of the channel conferring sensitivity to these agents are conserved among smooth muscle types. Activators of protein kinase C (PKC), however, were less effective at inhibiting $K_{\text{ATP}}$ currents in myocytes from gallbladder than from mesenteric artery. The phosphatase inhibitor okadaic acid increased the efficacy of PKC activators and revealed ongoing basal activation of $K_{\text{ATP}}$ channels by protein kinase A in gallbladder. These results suggest that phosphatases and basal kinase activity play an important role in controlling $K_{\text{ATP}}$ channel activity.

mesenteric artery; okadaic acid; electrophysiology; adenosine 5'-triphosphate-sensitive potassium channel

ATP-sensitive potassium ($K_{\text{ATP}}$) channels exhibit significant functional and pharmacological diversity, which reflects, in part, the molecular diversity of the channel structure. Functional $K_{\text{ATP}}$ channels are heteromultimers composed of four pore-forming inwardly rectifying $K^+$ channel (Kir) subunits of the Kir 6.0 subfamily (Kir 6.1 or 6.2) and four regulatory sulfonylurea receptor subunits (SUR1, 2A, or 2B) (4). Based on pharmacology, tissue distribution, and expression of recombinant $K_{\text{ATP}}$ channels, three broad classes of $K_{\text{ATP}}$ have been identified: pancreatic β-cell (Kir 6.2; SUR1) (7), cardiac-skeletal muscle (Kir 6.2; SUR 2A) (8), and smooth muscle $K_{\text{ATP}}$ channels. The molecular composition of smooth muscle $K_{\text{ATP}}$ channels is unclear because Kir 6.1, Kir 6.2, SUR 1, and SUR 2B have all been identified in smooth muscle (5, 9, 21).

The functional properties of $K_{\text{ATP}}$ channels in smooth muscle differ substantially from those in other cell types. Notably, they exhibit a high sensitivity to $K^+$ channel opening drugs such as pinacidil and levcromakalim and exhibit profound modulation by protein kinases (1, 2, 6, 11, 17, 24, 25). Smooth muscle relaxants [e.g., calcitonin gene-related peptide (CGRP) and adenosine], which act through stimulation of protein kinase A (PKA), activate $K_{\text{ATP}}$ channels in vascular smooth muscle (VSM; mesenteric artery (10, 18), coronary artery (15, 23), cerebral artery (11), and nonvascular smooth muscle (gallbladder)] (24, 25). Smooth muscle constrictors [e.g., neuropeptide Y, angiotensin II, serotonin, acetylcholine, and histamine] that act through stimulation of protein kinase C (PKC) have been shown to inhibit $K_{\text{ATP}}$ channels in mesenteric and cerebral arteries and esophageal and urinary bladder smooth muscle (1–3, 6, 11, 12). Indeed, PKA and PKC modulation of $K_{\text{ATP}}$ channels may be a significant mechanism for physiological and pathophysiological regulation of smooth muscle function. Despite the apparent similarity of $K_{\text{ATP}}$ channel function in various types of smooth muscle, there has been little information in terms of quantitative comparison of $K_{\text{ATP}}$ channels in this tissue. Uncovering diversity of smooth muscle $K_{\text{ATP}}$ channel function may point to avenues for the development of smooth muscle type-selective openers of $K_{\text{ATP}}$ channels that could have significant clinical implications.

We are particularly interested in the regulation of $K_{\text{ATP}}$ channels in gallbladder smooth muscle (GBSM). Modulation of $K_{\text{ATP}}$ channels in GBSM could provide a novel means for controlling gallbladder motility, which in turn would affect gallstone formation. $K_{\text{ATP}}$ channels within GBSM may be an important physiological target of CGRP, which is contained within sensory fibers in the gallbladder wall (13, 14). CGRP induces a membrane potential hyperpolarization and relaxation of intact gallbladder that is blocked by the $K_{\text{ATP}}$ channel inhibitor glibenclamide (25). CGRP causes gallbladder relaxation through activation of adenylyl cyclase, elevation of cAMP, stimulation of PKA, and activation of $K_{\text{ATP}}$ Channels (24). In contrast to VSM from mesenteric artery (18), the actions of CGRP on gallbladder appear to be limited by high levels of dephosphorylation by a phosphatase, such that $K_{\text{ATP}}$ currents immediately deactivate upon removal of CGRP (24). Although activators of PKC have been shown to cause pronounced inhibition of $K_{\text{ATP}}$ channels in smooth muscle from arteries (2, 11), esophagus (6), and urinary bladder (1), their effects on gallbladder are unknown.

The goal of this study was to determine the uniqueness of $K_{\text{ATP}}$ channels in GBSM, with the ultimate
hopes of designing tissue-selective approaches to modulating this channel. The first objective was to provide a pharmacological profile for key K⁺ channel openers (pinacidil and diazoxide) and glibenclamide on K<sub>ATP</sub> channel currents in isolated myocytes from gallbladder, and the second objective was to determine the effects of activators of PKC on these currents. We found that gallbladder K<sub>ATP</sub> channels resembled those of VSM with regard to their pharmacology. In contrast, however, gallbladder K<sub>ATP</sub> channels were much less sensitive to inhibition by PKC activators than those in VSM and responded differently to phosphatase inhibition.

**METHODS**

GBSM cell isolation. Guinea pigs between 2 and 4 wk old (250–350 g) and of either sex were euthanized with halothane and exsanguinated in a manner approved by the Institutional Animal Care and Use Committee of the University of Vermont. Gallbladders were dissected free from the liver and placed into ice-cold modified Krebs solution (in mM): NaCl 121, KCl 5.9, CaCl<sub>2</sub> 2.5, MgCl<sub>2</sub> 1.2, NaHCO<sub>3</sub> 25, NaH<sub>2</sub>PO<sub>4</sub> 1.2, and glucose 8 buffered to pH 7.4 with 95% O<sub>2</sub>-5% CO<sub>2</sub>. Gallbladders were transferred to Ca<sup>2+</sup>-free cell isolation solution containing (in mM): NaCl 55, monosodium glutamate 80, MgCl<sub>2</sub> 2, KCl 6, glucose 10, and HEPES 10 (adjusted to pH 7.3 with NaOH). Gallbladders were cut open longitudinally and pinned mucosal side up in a Sylgard-coated dish (Dow Corning, Midland, MI). The mucosa was removed with blunt forceps under a dissecting microscope. The remaining tissue was cut into small (1 × 3 mm) strips and placed into cell isolation solution containing: 1 mg/ml BSA, 1 mg/ml papain (23 U/mg; Worthington, Lakewood, NJ), and 1 mg/ml dithioerythritol (Sigma, St. Louis, MO). The mixture was incubated at 37°C for 30–35 min, and the tissue was subsequently transferred to a solution containing 1 mg/ml BSA, 1 mg/ml collagenase (1.01 U/ml; Fluka, Milwaukee, WI), and 100 µM CaCl<sub>2</sub> for a further 8–12 min. The tissue was rinsed in cold isolation solution and triturated with a glass Pasteur pipette to yield single smooth muscle cells. Cells were stored in glass vials on ice until required and used within 6 h of isolation.

Mesenteric artery smooth muscle cell isolation. Female Sprague-Dawley rats (12–14 wk old) were anesthetized with pentobarbital sodium (25 mg/kg), and the primary branch of the superior mesenteric artery was removed and dissected free from adipose tissue in cell isolation solution. The artery was cut open longitudinally and then enzymatically dissociated in cell isolation solution containing papain (0.5 mg/ml) and dithioerythritol (1 mg/ml) at 37°C for 40 min. The tissue was subsequently transferred to warmed cell isolation solution containing collagenase (0.7 mg/ml; Fluka) and 100 µM CaCl<sub>2</sub> for 10 min. The tissue was rinsed and triturated as before to produce isolated mesenteric arterial myocytes. For guinea pig mesenteric myocytes, cells were obtained as above, except arteries were initially incubated in 1 mg/ml papain and 1 mg/ml dithioerythritol at 37°C for 30 min, and then incubated in cell isolation solution containing collagenase (0.5 mg/ml; Sigma blended collagenase type F), 0.5 mg/ml hyaluronidase (Worthington, Lakewood, NJ), and 100 µM CaCl<sub>2</sub> for 10 min.

Patch-clamp recordings. Isolated smooth muscle cells suspended in cell isolation solution were placed into a recording chamber (1 ml vol) on the stage of an inverted phase-contrast microscope. Whole cell patch-clamp recordings were carried out as previously described (24) using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). Currents were sampled at 6.6 Hz with a Digidata A-D board attached to an IBM PC-compatible computer using Axotape 2 software (Axon Instruments). Electrodes were pulled from borosilicate glass (Sutter Instruments, Novato, CA), coated with dental wax, and fire polished to a final resistance of 3–8 MΩ.

Solutions and drugs. Electrodes were filled with a solution containing (in mM): KCl 102, KOH 38, NaCl 10, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 1, EGTA 10, Na<sub>2</sub>ATP 0.1, ADP 0.1, Na<sub>2</sub>GTP 0.2, glucose 10, and HEPES 10 adjusted to pH 7.2 with KOH. Cells were bathed in external solution containing (in mM): KCl 5, NaCl 135, MgCl<sub>2</sub> 1, HEPES 10, glucose 10, and CaCl<sub>2</sub> 0.1 (pH 7.4). When stable, the bathing solution was exchanged for a solution with the same composition as above, except that NaCl was substituted for KCl. All recordings described were performed at −60 mV in symmetrical 140 mM K⁺.

Pinacidil (RBI, Natick, MA) and glibenclamide (RBI) were prepared as 10 mM stock solutions in 100% DMSO, and diazoxide was prepared as a 100 mM stock in DMSO. Phorbol 12-myristate 13-acetate (PMA), 5-α-phorbol 12, 13-didecanoate (5αPDD), and 1,2-dioctanoyl-sn-glycerol (DOG) were prepared as 1 mM stock solutions in DMSO. Okadaic acid and adenosine 3', 5'-cyclic monophosphothioate (Rp-cAMPS; Calbiochem, San Diego, CA) were prepared as 100 µM and 10 mM stock solutions in DMSO and distilled water, respectively. Unless stated otherwise, all chemicals were purchased from Sigma.

Data analysis. All data are expressed as the mean ± SE of n cells. Glibenclamide-insensitive components of the current concentration response curve, was 1.4 µM, and Hill coefficient was 1.7.
were subtracted before analysis with a custom-written analysis program. Glibenclamide-sensitive currents were taken as a measure of K\textsubscript{ATP} currents (24). Concentration-response relationships were fitted with the equation 

\[ I = \frac{I_{\text{max}}}{1 + (K/D)^n} \]

where I is current, I\text{max} is maximal current, K is concentration of drug (D) required for half activation, and \( n \) is the Hill coefficient. Unpaired Student's t-tests were used to perform statistical analysis, and significance was reported at the 0.05 level.

RESULTS

Pharmacological modulation of K\textsubscript{ATP} currents in GBSM. Pinacidil is reasonably selective for smooth muscle, as an activator of K\textsubscript{ATP} channels (19). Diazoxide, in contrast, is equipotent in activating K\textsubscript{ATP} channels in smooth muscle and pancreatic \( \beta \)-cells, but is rather ineffective on K\textsubscript{ATP} channels in cardiac and skeletal muscle. The effects of these pharmacological fingerprints are unknown for K\textsubscript{ATP} channels in GBSM. We therefore tested the effects of pinacidil and diazoxide, as well as the classic inhibitor, glibenclamide, on K\textsubscript{ATP} currents in GBSM.

Whole cell K\textsubscript{ATP} currents were measured at a holding potential of -60 mV in symmetrical 140 mM K\textsuperscript{+}. Cells were dialyzed with a pipette solution containing 0.1 mM ATP, 0.2 mM GTP, and 0.1 mM ADP. Under these conditions, pinacidil activated K\textsubscript{ATP} currents in gallbladder myocytes in a concentration-dependent manner between 0.1 and 30 µM (Fig. 1A), with a Hill coefficient of 1.7 and EC\textsubscript{50} of pinacidil at 1.4 µM (Fig. 1B).

The K\textsubscript{ATP} channel opener diazoxide was a less potent activator of K\textsubscript{ATP} channels than pinacidil (Fig. 2A). The EC\textsubscript{50} concentration for diazoxide calculated from the mean concentration response data was 97.5 µM, and the Hill coefficient was 1.3 (Fig. 2B).

Glibenclamide inhibited K\textsubscript{ATP} currents elicited by 10 µM pinacidil with an IC\textsubscript{50} concentration of 77.6 nM, and Hill coefficient was 0.94 (Fig. 3A and B). Activators of PKC inhibit K\textsubscript{ATP} currents in smooth muscle cells from gallbladder and mesenteric artery. In vascular, urinary bladder, and esophageal smooth muscle, activators of PKC inhibit pinacidil-induced K\textsubscript{ATP} channel currents (1, 2, 6, 16, 20). The consequence of PKC stimulation on GBSM K\textsubscript{ATP} channels is not known. The activators of PKC, PMA (100 nM), and the membrane-permeable analog of diacylglycerol, DOG (1 µM), have been shown to inhibit K\textsubscript{ATP} currents in rabbit mesenteric artery by 86% and 87%, respectively (2). Similarly, 100 nM PMA inhibited K\textsubscript{ATP} currents in urinary bladder smooth muscle by 87% (1). Figure 4B and C, illustrate inhibition of K\textsubscript{ATP} currents in guinea pig mesenteric artery myocytes. K\textsubscript{ATP} currents were inhibited by 22.9 ± 6.4% (n = 6) with 100 nM DOG and 62.7 ± 5.9% (n = 6) with 1 µM DOG, respectively. K\textsubscript{ATP} currents were also inhibited by 100 nM (36.5 ± 3.1%,...
n = 5) and 1 µM (84.4 ± 2.9%, n = 6) DOG in rat mesenteric arteries. In contrast, 100 nM DOG had no significant effect on K<sub>ATP</sub> currents in gallbladder myocytes (44.9 ± 5.3 pA; control, 45.6 ± 6.3 pA; 100 nM DOG, n = 6). Increasing the DOG concentration to 1 µM, however, did inhibit gallbladder K<sub>ATP</sub> currents by 34.5 ± 10% (n = 6; Fig. 4, A and C).

PMA (100 nM) was also less effective in GBSM than VSM, reducing currents in gallbladder myocytes by only 45.7 ± 4% (n = 5; Fig. 5), compared with almost complete inhibition of K<sub>ATP</sub> currents in VSM (2, 11). The biologically inactive phorbol ester 4α-PDD had no measurable effect (n = 3) in GBSM, suggesting that the inhibition was mediated through PKC activation (Fig. 5). These results suggest that K<sub>ATP</sub> channels in GBSM are less sensitive to modulation by activators of PKC than those in VSM.

Actions of phosphatases on K<sub>ATP</sub> currents in GBSM and VSM. Decreased K<sub>ATP</sub> inhibition by PKC activators in GBSM could be due to a disruption in the PKC signaling pathway as a consequence of differences in K<sub>ATP</sub> channel structure or expression of different PKC isoforms, or, alternatively, could occur if the action of
PKC is counteracted by rapid dephosphorylation by phosphatases. Rapid dephosphorylation by phosphatases is supported by the observation that the deactivation of GBSM K\textsubscript{ATP} currents after removal of activators of PKA is rapid (24), compared with VSM (18).

To test the hypothesis that phosphatase activity plays an important role in the regulation of K\textsubscript{ATP} channels, the ability of DOG to inhibit K\textsubscript{ATP} currents in the presence of okadaic acid was examined in gallbladder and mesenteric artery myocytes. DOG-induced inhibition of K\textsubscript{ATP} currents was significantly enhanced in GBSM by okadaic acid, from 3.4 \pm 5.8\% (n = 5) to 29.9 \pm 9.0\% (n = 4) with 100 nM DOG, and from 34.5 \pm 10\% (n = 5) to 65.2 \pm 7.4\% (n = 6) with 1 \mu M DOG (Fig. 6, A and C). Similarly, okadaic acid increased inhibition of pinacidil-induced K\textsubscript{ATP} currents by 100 nM DOG in rat mesenteric artery smooth muscle from 36.5 \pm 3.1\% to 69.4 \pm 2\% (n = 5), and from 22.9 \pm 6.4\% (n = 6) to 56.6 \pm 15\% (n = 3) in guinea pig mesenteric arteries (Fig. 7, A and B). These results suggest that phosphatases play an important role in regulating the activity of K\textsubscript{ATP} channels in smooth muscle.

In addition to enhancing inhibition by PKC activators, application of okadaic acid alone increased K\textsubscript{ATP} currents in gallbladder myocytes. In the presence of pinacidil, okadaic acid caused a 51.6 \pm 10.5\% (n = 10) increase in the glibenclamide-sensitive inward current in GBSM (Fig. 6A). In the absence of pinacidil, K\textsubscript{ATP} currents were increased from 10.0 \pm 4.6 pA in control to 37.55 \pm 13.1 pA (n = 4) with 1 \mu M okadaic acid (Fig. 6B).

We investigated the possibility that this effect was a consequence of phosphatase inhibition. K\textsubscript{ATP} channels are under the dual regulation of PKA and PKC (2, 10, 11, 16), being inhibited by PKC activators and stimulated by PKA activators. Activators of cGMP-dependent protein kinase (PKG) have no effect on glibenclamide-sensitive currents in VSM (18, 23). Blocking phosphatases should increase the sensitivity of K\textsubscript{ATP} channels to both PKC and PKA activators. To investigate the possibility that the increase in the glibenclamide-sensitive current resulted from enhanced PKA activity, the effect of the PKA inhibitor Rp-cAMPs (100 \mu M) on K\textsubscript{ATP} currents was examined. Rp-cAMPs had no observ-
channels in rabbit mesenteric artery smooth muscle with an \(EC_{50}\) of 37 \(\mu\)M and a Hill coefficient of 1.0 (19) and in myocytes isolated from rat colon with an \(EC_{50}\) of 34.2 \(\mu\)M (17). In this study, glibenclamide inhibited gallbladder \(K_{\text{ATP}}\) currents with an IC50 of 77.6 nM and a Hill coefficient of 0.9, which is also comparable to the IC50 of 100 nM and the Hill coefficient of 0.8 reported for glibenclamide in VSM (19). The \(K^+\) channel openers pinacidil and diazoxide, and glibenclamide, are thought to bind to the SUR subunit of the \(K_{\text{ATP}}\) channel to modulate channel activity. This is supported by observations showing that recombinant \(K_{\text{ATP}}\) channels expressing different SUR subunits differ with regard to their sensitivity to pinacidil, diazoxide, and glibenclamide (7, 9).

Mutations that disrupt the integrity of two nucleotide binding folds (NBF1 and NBF2) of the SUR subunit abolish sensitivity of recombinant \(K_{\text{ATP}}\) channels to \(K^+\) channel-opening drugs (21). Kir 6.2 channels without SUR do not exhibit sensitivity to \(K^+\) channel-opening drugs (22), consistent with SUR being the target of these drugs. We have demonstrated here that \(K_{\text{ATP}}\) channels in GBSM have similar sensitivities to pinacidil, diazoxide, and glibenclamide as those in VSM and colon, suggesting that SUR is likely to be conserved among smooth muscle types. The molecular identity of SUR in smooth muscle, however, is unclear, because mRNA for both SUR1 and SUR 2B are found in VSM (3). In terms of their sensitivity to \(K^+\) channel openers and to glibenclamide, however, smooth muscle \(K_{\text{ATP}}\) channels resemble recombinant Kir 6.2/SUR 2B channels (9).

Inhibition of smooth muscle \(K_{\text{ATP}}\) channels by PKC: role of phosphatases. \(K_{\text{ATP}}\) channels in VSM are regulated by PKA and PKC. Vasoconstrictors such as histamine, serotonin, angiotensin II, acetylcholine, and neuropeptide Y that stimulate PKC cause a reduction in \(K_{\text{ATP}}\) channel activity (2, 11, 12), whereas vasodilators that stimulate PKA, such as CGRP and adenosine, activate \(K_{\text{ATP}}\) channels (10, 15, 18). Nitrovasodilators that activate PKG such as nitric oxide and sodium nitroprusside have not been demonstrated to activate \(K_{\text{ATP}}\) currents in VSM (18, 23). In non-VSM of the urinary bladder and esophagus, \(K_{\text{ATP}}\) channels are inhibited by activators of PKC (1, 6). The effects of PKC activators on \(K_{\text{ATP}}\) channels in GBSM were previously not known.

We have demonstrated here that GBSM \(K_{\text{ATP}}\) channels are less sensitive to inhibition by PKC activators than those in either guinea pig or rat mesenteric artery, suggesting there may be differences in \(K_{\text{ATP}}\) channel modulation in GBSM and VSM. The phosphatase inhibitor, okadaic acid, increased the effectiveness of PKC activators to inhibit \(K_{\text{ATP}}\) currents in GBSM and VSM, suggesting that phosphatases play an important role in regulating the activity of \(K_{\text{ATP}}\) channels. However, even in the presence of okadaic acid, PKC activators were more effective at inhibiting \(K_{\text{ATP}}\) channels in myocytes from mesenteric arteries than from gallbladder. This observation suggests that other factors in addition to phosphatases are responsible for the apparent difference in PKC activator efficacy.
Phosphatase inhibition activated a glibenclamide-sensitive current in GBSM that was blocked by the PKA inhibitor Rp-cAMPS (Fig. 6, A and D). In contrast, phosphatase inhibition by okadaic acid did not increase $K_{ATP}$ currents in myocytes from mesenteric arteries, but, in fact, slightly decreased the $K_{ATP}$ current (Fig. 7A). One explanation of these results is that PKA activation of $K_{ATP}$ currents is ongoing in GBSM, but not in VSM, and phosphatase inhibition enhances this activation by decreasing dephosphorylation of the PKA site. Our results suggest that, under physiological conditions, $K_{ATP}$ channel activity is dependent on a balance between phosphorylation by PKA and PKC, and dephosphorylation of their respective phosphorylation sites by phosphatases.

It is unknown what sites on $K_{ATP}$ channels or other unknown regulatory proteins are phosphorylated by PKA and PKC to cause physiological effects. There are several putative PKC phosphorylation sites on both SUR and Kir. Interestingly, there are three putative PKC phosphorylation sites at the COOH-terminal region of Kir 6.1 that are absent in Kir 6.2, making the Kir subunit a possible phosphorylation target of PKC, and may explain why there are tissue-specific differences in regulation of $K_{ATP}$ channels by PKC activators.

Similarly, there are four putative PKA phosphorylation sites on the $K_{ATP}$ Channel, two on SUR, and two on Kir.

In conclusion, we have demonstrated that gallbladder $K_{ATP}$ channels have a similar pharmacological profile as VSM $K_{ATP}$ channels with respect to their sensitivity to pinacidil, diazoxide, and glibenclamide. Furthermore, our results support a key role of phosphatases in the regulation of $K_{ATP}$ channel activity in GBSM and VSM.

We thank Gerald Herrera and George Wellman for critical appraisal of the manuscript.

This work was supported by National Institute of Neurological Disorders and Stroke Grant NS-26995, National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-45410, and National Heart, Lung, and Blood Institute Grant HL-44455. Address for reprint requests and other correspondence: G. M. Mawe, Dept. of Anatomy and Neurobiology, College of Medicine, Univ. of Vermont, Burlington, VT 05405 (E-mail: gmawe@uvm.edu).

Received 2 August 1999; accepted in final form 22 December 1999.

REFERENCES


