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G-Protein Regulation of an L-Type Calcium Channel Current in Canine Jejunal Circular Smooth Muscle

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Abstract. Calcium entry into smooth muscle cells is essential to maintain contractility. In canine jejunal circular smooth muscle cells the predominant calcium entry pathway is through L-type calcium channels. The aim of this study was to determine the G-protein regulation of L-type calcium channel current (I_{CaL}) in isolated canine jejunal circular smooth muscle cells. Barium (80 mM) was used as the charge carrier. GTP-γS and GTP increased maximal inward current from 118.7 ± 12 pA to 227.5 ± 21.5 pA ($n = 8$) and 174.6 ± 10.1 pA ($n = 6$) respectively. The increase in inward current was blocked by nifedipine suggesting it was through L-type calcium channels. Pertussis toxin did not alter baseline I_{CaL} while cholera toxin increased I_{CaL} from 125 ± 19 pA in controls ($n = 6$) to 347 ± 30 pA ($n = 4$). Staurosporine inhibited the increase in current evoked by GTP-γS and calyculin further increased I_{CaL} over the increase evoked by GTP-γS. The results suggest that cholera toxin sensitive G-proteins activate L-type calcium channels in isolated canine jejunal circular smooth muscle cells through protein phosphorylation.

Key words: G-proteins — Patch clamp — L-type calcium channels — Gastrointestinal smooth muscle — Canine

Introduction

Changes in intracellular free calcium (Ca^{2+}) are the final common pathway for contraction in both striated and smooth muscle [16]. A rise in intracellular Ca^{2+} initiates contraction and a fall initiates relaxation. There are two primary sources of free intracellular Ca^{2+}, entry of Ca^{2+} from the extracellular space and intracellular release from Ca^{2+} stores. In small intestinal circular smooth muscle, contraction can be initiated in the absence of extracellular Ca^{2+} indicating that release of Ca^{2+} from intracellular stores is sufficient to raise intracellular Ca^{2+} to the level necessary to trigger a contraction [23]. However, extracellular Ca^{2+} is essential to replete intracellular stores and sustain rhythmic contractions [23]. In the absence of extracellular Ca^{2+}, contractile activity diminishes and eventually ceases. The main calcium entry pathway for canine jejunal circular smooth muscle cells is through L-type Ca^{2+} channels, as nifedipine, an L-type Ca^{2+} channel blocker, blocks inward current [11]. Regulation of Ca^{2+} currents in smooth muscle has marked physiological relevance. An increase in Ca^{2+} entry results in an increase in intracellular Ca^{2+}. Ca^{2+} binds to calmodulin, promoting phosphorylation of myosin by myosin light chain kinase leading to contraction [16]. Ca^{2+} is also a ubiquitous second messenger, is involved in signal transduction pathways and in the regulation of several ion channels that modulate membrane potential [2].

L-type Ca^{2+} channel current (I_{CaL}) in canine jejunal circular smooth muscle is regulated by the neurotransmitter acetylcholine and by the gastrointestinal hormone motilin [7, 8, 11]. Motilin increases Ca^{2+} entry through L-type Ca^{2+} channels in both canine and human jejunal circular smooth muscle [7, 8]. Little is known about the signal transduction mechanism involved in the actions of motilin on gastrointestinal smooth muscle at a cellular level but recent data suggest that the motilin receptor may be G-protein coupled [4]. GTP-binding regulatory proteins are divided into two major classes, heterotrimeric G-proteins, which consist of α, β and γ subunits, and low molecular weight monomeric G-proteins [1, 22]. Heterotrimeric G-proteins are usually associated with the plasma membrane and are linked to cell membrane receptors, including ion channels [15]. G-proteins regulate

ion channel function in many cell types, including smooth muscle [reviewed in 19]. Both direct modulation of ion channel function by G-proteins as well as an indirect modulation through phosphorylation have been reported [reviewed in 3].

The aim of this study was therefore to determine the effect of G-protein activation and protein phosphorylation on I_{CaL} in isolated canine jejunal circular smooth muscle cells.

Materials and Methods

Single, isolated, circular smooth muscle cells were obtained from the jejunum of adult mongrel dogs of either sex. The use of canine jejunum was approved by the Institutional Animal Care and Use Committee. Dogs were euthanized with an overdose of barbiturate (45 mg/Kg) and a 10-cm piece of jejunum removed just distal to the ligament of Treitz. The dissociation procedure used to obtain single, relaxed, circular smooth muscle cells was as previously described [9, 10]. In brief, full thickness strips of jejunum were pinned to the floor of a dissecting dish over ice and incisions were made parallel to the longitudinal muscle axis extending to but not into the circular muscle layer. The serosa and longitudinal muscle layer were removed leaving circular muscle and submucosa. Incisions were made parallel to and through the circular muscle axis. Strips of circular muscle were gently peeled off of the submucosa, placed in modified Hanks solution (Sigma #H8389) and cut into 2-mm pieces. They were placed in 8 ml of Hanks solution containing 15 mg of papain (Sigma #P4762) and 3.1 mg of dithiothreitol (Sigma #D0632) and gently stirred for 20 min at 37°C. After centrifugation to remove the enzyme solution, the tissue was transferred to fresh Hanks solution and mechanically dissociated at 37°C to obtain single relaxed circular smooth muscle cells.

Patch clamp recordings were made using an Axopatch 200A voltage clamp amplifier (Axon Instruments, Foster City, CA) connected to a TL-1 Labmaster driven by PClamp software. Whole cell recordings were obtained using Kimble KG-12 glass pulled on a P-80 puller (Sutter Instruments, Novato, CA). Electrodes were coated with R6101 (Dow Corning, Midland, MI) and fire polished to a final resistance of 3 to 5 MΩ. Records were obtained using standard whole-cell recording techniques. Three runs were averaged for each recording unless otherwise noted. Records were sampled at 2 kHz and filtered at 1 kHz. Data were analyzed with Clampfit or custom macros in Excel (Microsoft, Redmont, WA) and comparisons between currents made at peak measured values. Paired students *t* test was used to evaluate statistical significance. All records were obtained at room temperature (22°C).

Drugs and Solutions

Nifedipine, staurosporine and calyculin were obtained from Sigma Chemical (St. Louis, MO). Barium chloride was obtained from Fisher (Springfield, NJ), and GTP-γS, GDP-βS, pertussis toxin and cholera toxin from Calbiochem (San Diego, CA). Drugs were applied by complete bath changes with the solution containing the drug or introduced inside the cells through the intracellular solution. The time for a complete solution exchange (4× the bath volume) was approximately 30 sec. Stock solutions of staurosporine and calyculin were made up in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was <1:1000. All other solutions were made up on the day of use without solvents. The intracellular solution contained (in mM) Cs^+ 150, Cl^- 20, EGTA 2, hepes 5, methanesulfonate 130. The barium solution contained (in mM) Ba^{2+} 80, Cl^- 160, hepes 5.

Results

To minimize outward potassium (K^+) current, the intracellular solution contained 150 mM cesium (Cs^+) to replace intracellular K^+ and block K^+ channels. To maximize inward current 80 mM Ba^{2+} was used as the charge carrier as Ba^{2+} is more permeant through Ca^{2+} channels than Ca^{2+} itself. The mean shift in membrane potential due to the surface charge screening effects of 80 mM Ba^{2+} was measured by comparing current-voltage records obtained with 80 mM Ba^{2+} as the charge carrier to records obtained with 10 mM Ba^{2+}. The mean shift was +25 mV.

Effect of G-Protein Activation on the Inward Current

The effects of G-protein activation on inward current were examined in isolated canine jejunal circular smooth muscle cells using GTP-γS, a nonhydrolyzable analogue of GTP. Freshly dissociated cells were divided into two aliquots and GTP-γS (200 μM) was introduced into one set of cells via the intracellular pipette solution after obtaining traditional whole cell access. The other set of cells served as controls, without GTP-γS in the pipette solution. Cells were held at −70 mV and the membrane potential pulsed in 16 steps from −50 to +70 mV. Each voltage step was 350 msec long and the interpulse interval was 1 sec to allow complete recovery from inactivation. The maximal inward current recorded in control cells was 118.7 ± 12 pA, ($n = 8$). The maximal inward current recorded from cells with GTP-γS (200 μM) in the intracellular solution was 227.5 ± 21.5 pA ($n = 8$, $P < 0.05$, Fig. 1). The dihydropyridine nifedipine (1 μM), an L-type Ca^{2+} channel blocker, completely inhibited inward current in the presence of GTP-γS (200 μM, $n = 4$) suggesting that the increase in inward current was due to activation of the baseline I_{CaL} (Fig. 2). Maximal inward current was present within 60 sec of establishing intracellular access. A more accurate estimate of the time to maximal activation of the inward current could not be obtained due to the duration of the pulse protocol used and the unknown time required for GTP-γS to diffuse into the cells.

Activation times were calculated for a range of voltages in the presence and absence of GTP-γS. Activation times for both control cells and cells exposed to GTP-γS were well fit with a single exponential. No difference in activation times was noted (Fig. 3). τ for activation at maximal inward current (+22 mV) was 6.8 ± 0.9 msec for control cells. This value was in close agreement with

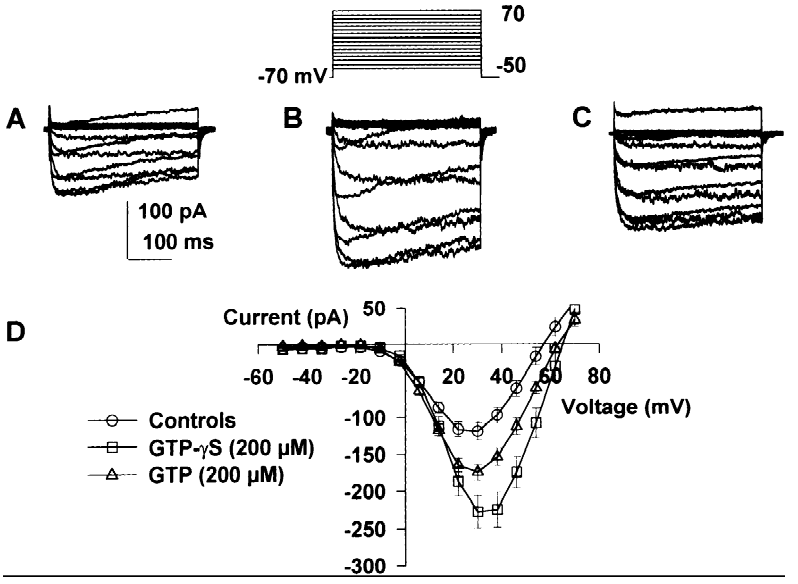

Fig. 1. Effect of GTP-γS (200 μM) and GTP (200 μM) on inward currents carried by 80 mM Ba in canine jejunal circular smooth muscle cells. Panel *A* shows typical currents recorded in the absence of GTP-γS, panel *B* typical currents recorded in the presence of GTP-γS, and panel *C* typical currents recorded in the presence of GTP. Panel *D* shows the mean *I-V* relationships. The mean maximal inward current in control cells was 118.7 ± 12 pA ($n = 8$) in the presence of GTP-γS, 227.5 ± 21.5 pA ($n = 8$, $P < 0.05$), and in the presence of GTP, 174.6 ± 10.1 pA ($n = 6$, $P < 0.05$). Note in this and in Figs. 2, 4–8 the *I-V* relationships are not leak subtracted nor adjusted for the charge screening effect of Ba.

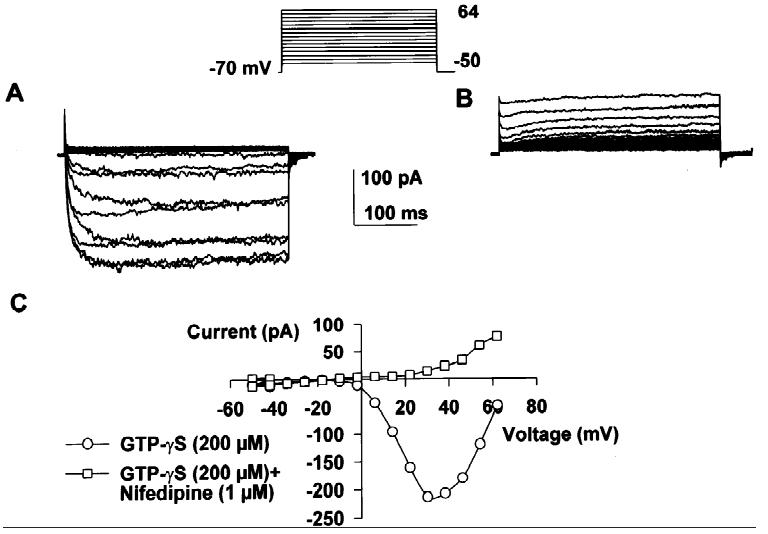

Fig. 2. Effect of the L-type calcium channel blocker nifedipine on inward currents carried by 80 mM Ba in a canine jejunal circular smooth muscle cell. Panel *A* shows typical currents recorded in the presence of GTP-γS (200 μM) and panel *B* the currents recorded from the same cell in the presence of GTP-γS (200 μM) and nifedipine (1 μM). Panel *C* shows the *I-V* relationships. Maximal inward current in the presence of GTP-γS was 217 pA. Nifedipine completely blocked the inward current suggesting that GTP-γS activated the baseline I_{CaL} current found in these cells.

previously reported values [11]. Similarly, τ for activation was 7.3 ± 0.5 msec for cells exposed to GTP-γS (200 μM, $n = 8$, $P > 0.05$).

As a nonhydrolyzable analogue of GTP, GTP-γS, increased inward current, the effects of GTP on inward current were also tested. GTP (200 μM) was introduced into the cells via the intracellular solution. In control cells unexposed to GTP, maximal inward current was 118.7 ± 12 pA (same control cells as above, $n = 8$), and in cells exposed to GTP 174.6 ± 10.1 pA ($n = 6$, $P < 0.05$, Fig. 1). The data suggest that GTP, like GTP-γS, also increased inward current in canine jejunal circular smooth muscle cells.

To determine if the G-protein(s) involved belonged to the Go\Gi subclass, the effects of the Go\Gi G-protein inhibitor pertussis toxin were tested (Fig. 4). Freshly dissociated cells were divided into two aliquots and one set incubated with pertussis toxin (240 ng/ml) for 2–4 hr while the other set exposed to the same solution without pertussis toxin. The order in which cells were patch clamped was altered on different days. Pertussis toxin was without effect on inward current (135.7 ± 31 pA *vs.* 116 ± 14 pA in control cells, $n = 8$, $P > 0.05$). The effects of cholera toxin on inward current were tested by incubating cells with cholera toxin (100 ng/ml) for 2 hr and then recording inward current. Control cells were patch clamped without exposure to cholera toxin. The maximal inward current in the presence of cholera toxin was 347 ± 30 pA ($n = 4$) compared to 125 ± 19 pA ($n = 6$) in the absence of cholera toxin ($P < 0.05$, Fig. 5).

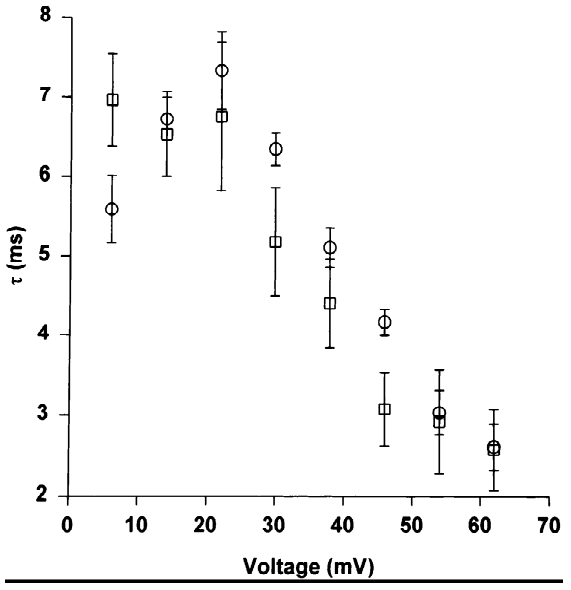

Fig. 3. Effect of GTP-γS on activation time (τ) at several voltages. Activation was well fit with a single exponential. No difference in τ was noted between control canine jejunal circular smooth muscle cells (○, $n = 8$) and cells (□, $n = 8$) exposed to GTP-γS (200 μM, $P > 0.05$ at all voltages).

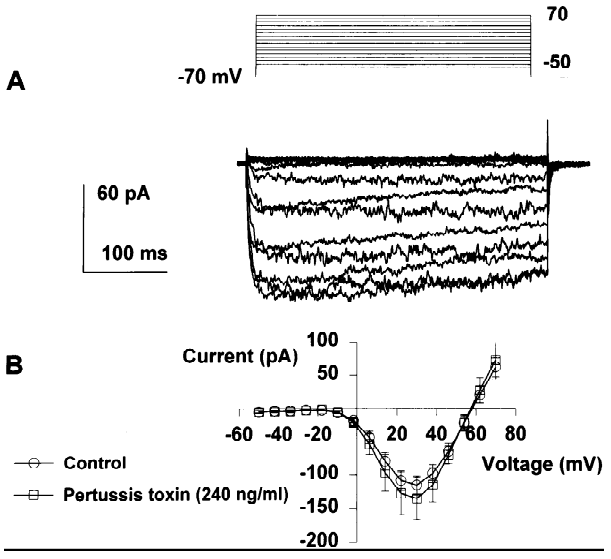

Fig. 4. Effect of pertussis toxin on inward currents carried by 80 mM Ba. Panel *A* shows typical inward currents recorded from a canine jejunal circular smooth muscle cell incubated with pertussis toxin. Panel *B* shows the mean *I-V* relationships obtained from 6 cells showing the lack of effect of pertussis toxin on inward current. Solutions containing canine jejunal circular smooth muscle cells were divided into 2 aliquots and one aliquot incubated with pertussis toxin (240 ng/ml) for 2–4 hr while the other aliquot served as a control. The maximal inward current recorded from control cells was 116 ± 14 pA and from cells exposed to pertussis toxin 135.7 ± 31 pA ($n = 6$, $P > 0.05$).

The data suggest that cholera toxin sensitive G proteins (Gs subclass) were involved in the increase in I_{CaL} evoked by GTP and GTP-γS in canine jejunal circular smooth muscle cells.

Effect of G-Protein Inhibition on the Inward Current

To determine the effects of G protein inhibition on inward current, cells were patch clamped in the presence of the G-protein inhibitor GDP-βS. GDP-βS (100 μM) in the intracellular solution had no effect on maximal inward current as compared to control cells. Maximal inward current recorded in the presence of GDP-βS (100 μM, $n = 8$) was 113.4 ± 16 compared with 101 ± 18 pA in control cells ($n = 8$, $P > 0.05$, Fig. 6). The results suggest that there is little, if any, regulation of I_{CaL} by G-proteins in the unstimulated state.

Effect of Phosphorylation State on the Inward Current

The mechanism of the stimulatory action of G-proteins on I_{CaL} differs in different cell types. Both a direct effect of G-proteins on I_{CaL} and an indirect effect secondary to protein phosphorylation have been described [6, 13, 14]. To address the mechanism of action the effects of the state of protein phosphorylation on I_{CaL} were tested in isolated canine jejunal circular smooth muscle cells. To alter the cellular balance of phosphorylation towards the dephosphorylated state, cells were exposed to the non-specific protein kinase inhibitor staurosporine. Cells were exposed to staurosporine (500 nM) for 15 min and then patch clamped in the presence of staurosporine, while control cells were patch clamped without exposure to staurosporine. In both sets of cells GTP-γS (200 μM) was introduced into the cells through the intracellular solution to activate intracellular G-proteins. Maximal inward current recorded in control cells not exposed to staurosporine was 146.2 ± 16 pA compared with 100.3 ± 10.2 pA in the presence of staurosporine ($n = 6$, $P < 0.05$, Fig. 7). The effects of protein phosphorylation on I_{CaL} were tested using the phosphatase type 1 and type 2 A and B inhibitor calyculin A. Control cells were patch clamped in the absence of GTP-γS or calyculin. The other cells were patch clamped in the presence of GTP-γS (200 μM) in the recording pipette and after maximal inward current was recorded, calyculin (100 nM) was added to the bath. Maximal inward current for control cells was 92 ± 3.6 pA ($n = 6$). Maximal inward current in the presence of GTP-γS was 188 ± 35 pA and increased to 245 ± 43 pA in the presence of calyculin ($n = 4$, all $P < 0.05$, Fig. 8). The increase in current evoked by calyculin A was not sustained. Four minutes after

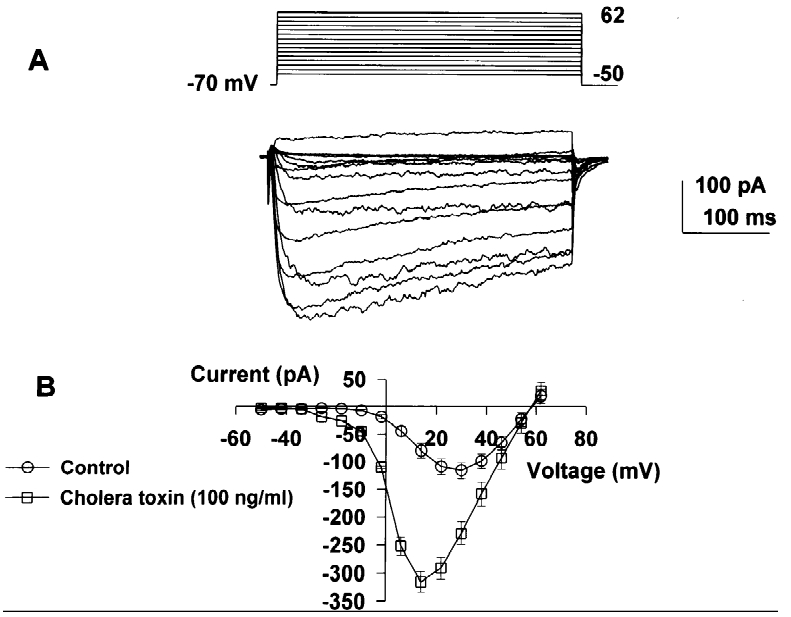

Fig. 5. Effect of cholera toxin on inward currents carried by 80 mM Ba. Panel *A* shows typical inward currents recorded from a canine jejunal circular smooth muscle cell incubated with cholera toxin. Panel *B* shows the mean *I-V* relationships obtained from 4 cells showing the stimulatory effect of cholera toxin on inward currents. Solutions containing canine jejunal circular smooth muscle cells were divided into 2 aliquots and one aliquot incubated with cholera toxin (100 ng/ml) for 2 hr while the other aliquot served as a control. The maximal inward current recorded from control cells was 125 ± 19 pA and from cells exposed to cholera toxin 347 ± 30 pA ($n = 4$, $P < 0.05$) suggesting that the inward current was cholera toxin activated.

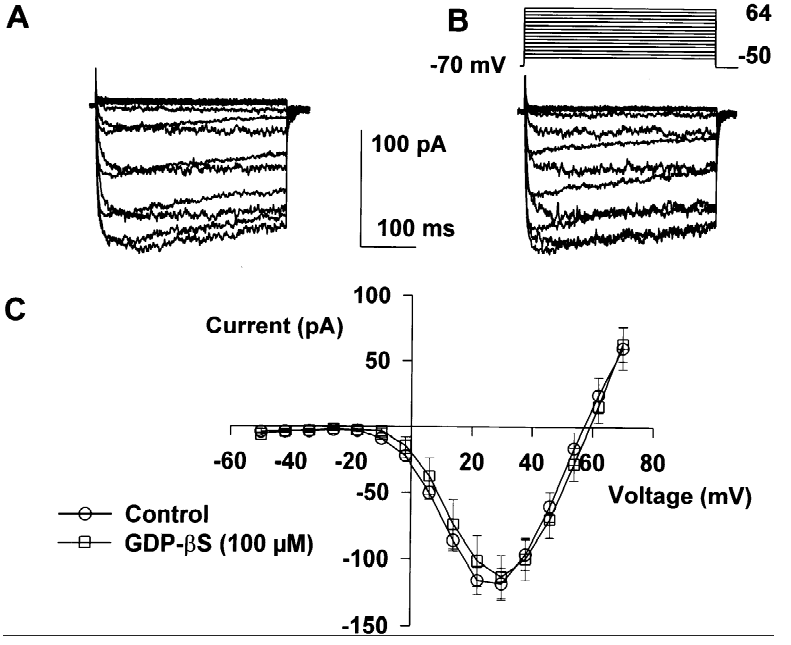

Fig. 6. Effect of G-protein inhibition on inward currents carried by 80 mM Ba recorded from canine jejunal circular smooth muscle cells. Panel *A* shows typical control currents recorded in the absence of GDP-βS and panel *B* typical currents recorded in the presence of GDP-βS (100 µM). Panel *C* shows the mean *I-V* relationships obtained from 8 cells. The mean maximal inward current in control cells was 101 ± 18 pA ($n = 8$) and in the presence of GDP-βS 113.4 ± 16 pA ($n = 8$, $P > 0.05$) suggesting that there was no regulation of the inward current by G-proteins under 'resting' conditions.

peak inward current was recorded, inward current in the presence of calyculin A decreased by 49 ± 6.7% ($n = 4$).

Discussion

This study reports that, in isolated canine jejunal circular smooth muscle cells, G-protein activation led to augmentation of an inward current carried through L-type Ca^{2+} channels. Activation of I_{CaL} was most likely secondary to a change in protein phosphorylation as staurosporine, the nonspecific protein kinase inhibitor, inhibited the effects of G-protein activation on I_{CaL} and calyculin, a phosphatase inhibitor, accentuated the effects of G-protein activation on I_{CaL}. The study, however, does not exclude a direct modulation of L-type Ca^{2+} channel gating by G-proteins. To prove direct G-protein modulation a purified ion channel has to be shown to be directly gated by a purified G-protein subunit and direct physical association between the channel and the G-protein must be shown [3]. This criterion is not easily met. Even in excised patches various cytoplasmic cellular components

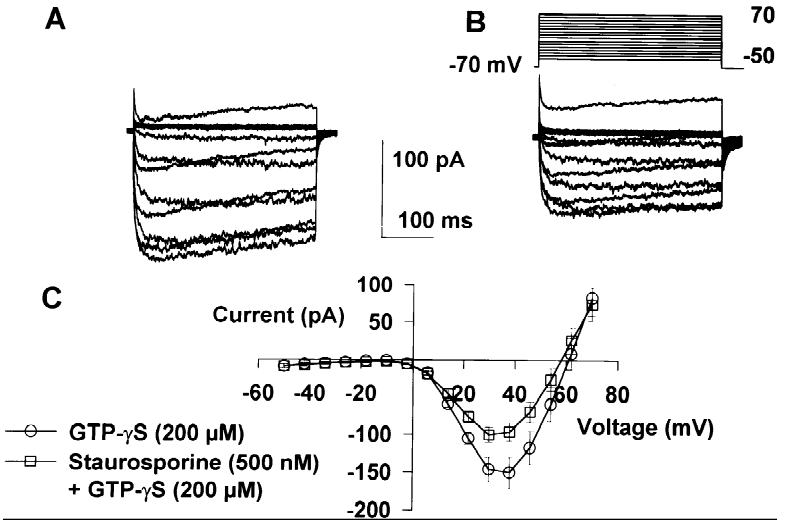

Fig. 7. Effect of the nonspecific protein kinase inhibitor, staurosporine, on inward currents carried by 80 mM Ba. Panel *A* shows typical control currents recorded from an isolated canine jejunal circular smooth muscle cell and panel *B* typical currents recorded in the presence of staurosporine. Mean *I-V* relationships obtained from 6 cells are shown in panel *C*. Solutions containing canine jejunal circular smooth muscle cells were divided into 2 aliquots and one aliquot incubated with staurosporine (500 nM) for 15 min while the other aliquots served as a control. Both sets of cells were patch clamped in the presence of GTP-γS (200 μM) in the pipette solution. The mean maximal inward current in control cells was 146.2 ± 16 pA ($n = 6$) and in the presence of staurosporine 100.3 ± 10.2 pA ($n = 6$, $P < 0.05$).

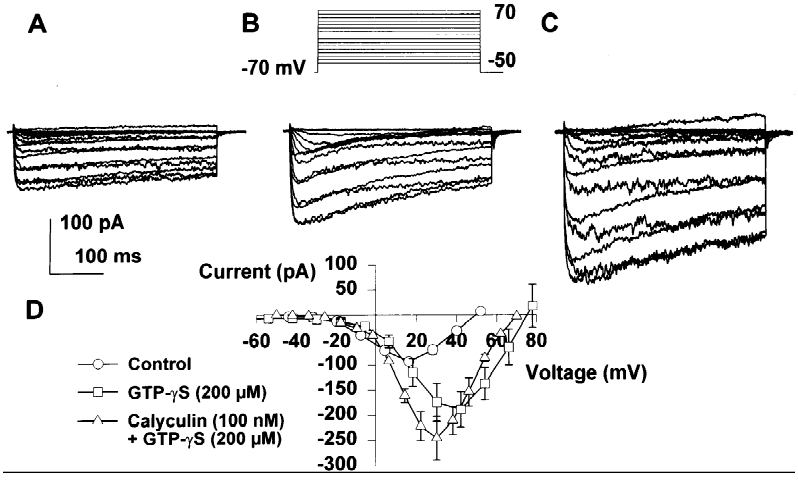

Fig. 8. Effect of the phosphatase inhibitor calyculin on inward currents carried by 80 mM Ba in canine jejunal circular smooth muscle cells. Cells were patch clamped with GTP-γS (200 μM) in the recording pipette. After maximal inward current was recorded, calyculin A (100 nM) was added to the bath solution. Control cells were patch clamped without GTP-γS or calyculin. Panel *A* shows typical currents recorded from a control cell. Panel *B* shows maximal inward currents recorded from a canine jejunal circular smooth muscle cell in the presence of GTP-γS and panel *C* the current recorded from the same cell in the presence of GTP-γS and calyculin A. In this cell maximal inward current increased by 59% in the presence of calyculin. Panel *D* shows mean *I-V* relationships obtained from 4 cells. The mean maximal inward current in control cells was 92 ± 7.6 pA ($n = 8$), in the presence of GTP-γS only, 188 ± 21.5 pA and in the presence of GTP-γS and calyculin A 245 ± 43 pA ($n = 4$, all $P < 0.05$).

are still attached to the excised membrane [24]. Furthermore, L-type Ca^{2+} channels rapidly run down in excised patches making excised patch experiments difficult to perform and interpret.

In all experiments carried out for this report, Ba^{2+} was used as the charge carrier. Ba^{2+} was used to maximize inward current as, even with Ba^{2+} as the charge carrier, the whole cell inward current in canine jejunal circular smooth muscle is only about 2.4 pA/pF [11]. The use of Ba^{2+} may however have masked any potential downregulatory effects of the increased Ca^{2+} entry (with Ca^{2+} as the charge carrier) evoked by G-protein activation of L-type Ca^{2+} channels. Increased intracellular Ca^{2+} may have effects on the L-type Ca^{2+} channel itself or on other regulatory mechanisms that control I_{CaL}. Several phosphatases are Ca^{2+} dependant and may modulate Ca^{2+} channels activity [12].

G-protein modulation of Ca^{2+} channel function has been well described in the literature [reviewed in 5, 20]. In many neurons and secretory cells the main effect of G-protein activation is inhibition of Ca^{2+} channels through a pertussis toxin sensitive G-protein pathway. The Ca^{2+} channel current inhibited by this pathway is predominantly N-type although there is some effect on L-type Ca^{2+} channels [reviewed in 5]. In the heart, both activation and inhibition of L-type Ca^{2+} channels by G-proteins has been reported [reviewed in 20]. Activation of L-type Ca^{2+} channels in heart is through a cholera toxin activated, pertussis toxin insensitive, G-protein pathway. It appears that the L-type Ca^{2+} channel present in canine jejunal circular smooth muscle is regulated by a similar G-protein pathway.

There is evidence in the literature for both a direct 'membrane delimited' and an indirect effect of G-proteins on I_{CaL}. In bovine adult freshly dissociated trachea smooth muscle cells I_{CaL} was activated by GTP-γS

but not modulated by adenosine 3′,5′-cyclic monophosphate (cAMP), cAMP analogues or the catalytic subunit of cAMP-kinase suggesting a direct effect [18]. In heart smooth muscle a direct effect of G-proteins on I_{CaL} has been reported [14] but the majority, if not the entire effect on I_{CaL}, can be accounted for by channel phosphorylation [13]. The exact nature of the protein kinase involved in modulation of I_{CaL} in canine jejunal circular smooth muscle cells was not addressed in this study but a recent report examining the effects of cAMP on I_{CaL} in canine colonic circular smooth muscle cells suggests that a cAMP-dependent protein kinase may be involved in the pathway [17].

The data on the effects of phosphorylation on I_{CaL} presented in this study differ from a previous report in colonic and gastric smooth muscle cells where calyculin and okadaic acid, phosphatase inhibitors, were found to inhibit I_{CaL} [25]. However, a subsequent report by the same authors suggests that low concentrations of cAMP activate a cAMP-dependent protein kinase that phosphorylates a site on L-type Ca^{2+} channels while higher concentrations of cAMP or cGMP-dependent protein kinases phosphorylate further sites on L-type Ca^{2+} channels leading to inhibition of L-type Ca^{2+} channels [17]. The data presented in this report are in agreement with this hypothesis, as calyculin A had a biphasic action on I_{CaL}, first activating I_{CaL}, then inhibiting I_{CaL}.

G-proteins have been previously shown to participate in receptor-mediated contraction of isolated intestinal smooth muscle cells. GTP-γS, in guinea pig circular and longitudinal intestinal smooth muscle, increased inositol-1,4,5-triphosphate (IP_3), mobilized intracellular Ca^{2+} and elicited a concentration dependent contraction. The Ca^{2+} channel blocker methoxyverapamil inhibited the increase in intracellular Ca^{2+} in longitudinal but not circular guinea pig smooth muscle cells [21]. The present study suggests that, in canine jejunal circular smooth muscle cells, G-protein activation may also increase Ca^{2+} entry through L-type Ca^{2+} channels as well as mobilize intracellular Ca^{2+}.

In the present study pertussis toxin was without effect on baseline I_{CaL}. However, GDP-βS was without effect on I_{CaL} suggesting that there was little if any baseline regulation of I_{CaL} by G-proteins. Therefore, to correctly determine the effects of pertussis toxin on I_{CaL}, I_{CaL} needs to be activated by endogenous G-proteins. We have previously shown that motilin, a gastrointestinal peptide, activates I_{CaL} through a pertussis toxin insensitive G-protein coupled pathway [8]. The combined data of the motilin study and the present study suggest that I_{CaL} was regulated by pertussis toxin insensitive, cholera toxin sensitive G-proteins.

In summary, I_{CaL} in canine jejunal circular smooth muscle cells was G-protein regulated. G-protein activation by GTP-γS, GTP or cholera toxin increased I_{CaL}. Phosphorylation modulated the effects of G-protein activation on I_{CaL} suggesting that the effects of G-proteins was secondary to protein phosphorylation.

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