Dual concentration-dependent effects of phorbol 12, 13-dibutyrate on spontaneous and acetylcholine-induced electrical responses recorded from isolated circular smooth muscle of the guinea-pig stomach antrum

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Abstract

Intracellular recordings of electrical activity were made from circular smooth muscle cells in small segments of tissue isolated from the guinea-pig stomach antrum. Every cell that was impaled exhibited a rhythmic generation of slow potentials. Experiments were carried out to test the effects of three different concentrations (1, 10 and 100 nM) of phorbol 12, 13-dibutyrate (PDBu) on these slow potentials and on the responses produced by acetylcholine (ACh), in the presence of nifedipine and Nω-nitro-L-arginine (nitroarginine), known inhibitors of L-type Ca-channels and nitric oxide synthase, respectively. The resting membrane potential was –62 ± 7 mV, while the frequency and amplitude of the slow potentials were 1.6 ± 0.1 cycle per min (cpm) and 33 ± 1 mV, respectively. Application of 1 nM PDBu increased the frequency of slow potentials, with no significant change in the membrane potential and amplitude of slow potentials. At a concentration of 100 nM, PDBu depolarized the membrane by about 6 mV, and either decreased the amplitude and frequency of the slow potentials or abolished them. The amplitude and frequency of the slow potentials were not significantly changed in the presence of 10 nM PDBu. In the presence of chelerythrine (1–2 µM), a known inhibitor of protein kinase C (PKC), the increase in frequency of slow potentials by 1 nM PDBu and depolarization produced by 100 nM PDBu were not elicited. The increase in frequency of slow potentials by 100 nM ACh was inhibited by PDBu, in a concentration-dependent manner, and ACh-responses were abolished in the presence of 100 nM PDBu. These results indicate that PDBu has dual actions on the spontaneous activity of antral circular muscle, with low concentrations increasing and high concentrations inhibiting the frequency of the slow potentials. The former may be produced by activation of protein kinase C (PKC). As the ACh-induced excitation of slow potentials is inhibited by PDBu, a possible causal relationship between the inhibition and over-activation of PKC is considered.

Key words: slow potential, protein kinase C, phorbol ester, acetylcholine, gastric muscle
Introduction

Gastric smooth muscle spontaneously generates slow waves and spike potentials (Tomita, 1981). The latter potentials may be produced by activation of voltage-gated Ca-channels, since these potentials are blocked by Ca-antagonists (Golenhofen and Lammer, 1972; Ishikawa et al., 1985; Ozaki et al., 1991; Tomita et al., 1998). These reports also indicate that Ca-antagonists cannot block the generation of slow waves. Examination of the effects of different combinations of ionic composition revealed that slow waves are inhibited only in solutions containing no Ca\(^{2+}\), which indicates the importance of Ca\(^{2+}\) in the generation of slow waves (Tomita, 1981).

Thuneberg (1982) speculated that interstitial cells of Cajal distributed in the myenteric layer of the gastric wall (ICC-MY) are the pacemaker cells for gastric motility, since these cells are rich in mitochondria and form gap junctional connections with other ICC-MY and smooth muscle cells. Hara et al. (1986) suggested that in the dog colon the pacemaker cells responsible for the spontaneous electrical activity are distributed in regions close to the myenteric layer, since the amplitude of the spontaneous activity is greater in cells distributed closer to this layer. The absence of slow waves in the intestine of mutant mice that have lost expression of the c-kit gene, suggests that cells expressing c-Kit protein (such as ICC-MY) are essential for the generation of slow waves (Ward et al., 1994; Huizinga et al., 1995). A series of experiments carried out following these important findings indicates that ICC-MY may be the pacemaker cells for spontaneous activity in gastrointestinal activity (Sanders, 1996; Huizinga et al., 1997; Sanders et al., 1999; Suzuki, 2000; Hirst et al., 2003; Kito and Suzuki, 2003; Takaki, 2003). In the smooth muscle of the guinea-pig stomach, excitation of ICC-MY precedes that of the smooth muscle cells, indicating directly that ICC-MY are indeed producing pacemaker potentials for slow waves (Dickens et al., 1999). In circular smooth muscle bundles isolated from the guinea-pig stomach antrum, regenerative potentials (slow potentials) appear periodically (Suzuki and Hirst, 1999), indicating that interstitial cells of Cajal distributed in the smooth muscle bundles (intramuscular interstitial cells of Cajal, ICC-IM) are also capable of a pacemaker role. Slow potentials, which may be formed by the summation of unitary potentials generated in ICC-IM (Edwards et al., 1999), can be abolished by low concentrations of caffeine (0.5–1 mM), while slow waves can not (Suzuki and Hirst, 1999; Fukuta et al., 2002; Nose et al., 2000; Kito et al., 2002a). Although the cellular mechanism involved in the selective inhibition of slow potentials by caffeine remains unclear, the inhibition appears with no alteration to the tissue level of cyclic AMP concentration (Nakamura et al., 2004c), thereby suggesting that the inhibition is independent of the increased cAMP level.

Slow potentials generated in the circular muscle of the guinea-pig stomach antrum have a refractory period for their generation (Nose et al., 2000). A possible involvement of protein kinase C (PKC) in the refractory period for the evoked slow potentials has been indicated (Kito et al., 2002b; Suzuki et al., 2002a). PKC is activated by diacyl glycerol (Nishizuka, 1992), and the possible involvement of this pathway in determining the frequency of slow potentials is indicated by experiments using an inhibitor of diacyl glycerol lipase (Suzuki et al., 2002b). An increase in the frequency of slow potentials during activation of muscarinic receptors with acetylcholine (ACh) (Nakamura and Suzuki, 2004b) or by excitation of cholinergic nerves (Lee
et al., 2004) is also causally related to the activation of PKC in smooth muscle of the guinea-pig stomach. The refractory period for slow potentials is reduced by stimulating muscarinic receptors with ACh through activation of PKC (Kito et al., 2002b). This suggests that the increase in frequency of slow potentials caused by ACh involves enhanced activity of PKC. On the other hand, the increase in the amplitude of slow potentials during excitation of smooth muscle with ACh is accompanied by an enhanced release of Ca\(^{2+}\) from internal stores through IP\(_3\) receptors (Nakamura and Suzuki, 2004b). Thus, both IP\(_3\) and PKC may be important in the regulation of different aspects of slow potentials.

The present experiments were carried out to observe the effects of phorbol 12, 13-dibutyrate (PDBu) on the spontaneous activity of circular smooth muscle isolated from the guinea-pig stomach antrum. Phorbol esters including PDBu are known activators of PKC (Nishizuka, 1984, 1992). The aim of the present experiments was therefore focused on the effects of PDBu on the amplitude and frequency of slow potentials generated spontaneously and also those elicited during stimulation with ACh. The results indicated dual effects of PDBu on both the amplitude and frequency of slow potentials, with excitation at low concentrations and inhibition at high concentrations. The excitatory actions of ACh on slow potentials were also inhibited by PDBu.

Some of these results were reported briefly to the 46th Annual Meeting of the Japan Society of Smooth Muscle Research held in Tokyo, Japan (Nakamura and Suzuki, 2004a).

**Materials and Methods**

Male albino guinea-pigs, weighing 200–550 g, were anaesthetized with fluoromethyl 2,2,2-trifluoro-1-(trifluoromethyl) ethyl ether (Sevoflurane; Maruishi Pharmaceutical, Osaka, Japan) and decapitated. All animals were treated ethically according to the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences, approved by The Physiological Society of Japan. The stomach was excised and opened by cutting along the small curvature in Krebs solution. The mucosal layer was removed by cutting with fine scissors, and a block of circular smooth muscle bundles was isolated from the antrum region. A segment of single circular muscle bundles (80–100 µm wide and 200–300 µm long) was prepared by mechanical removal of the longitudinal muscle layer with attached myenteric layers, using fine forceps. The preparation was pinned out on a Sylgard plate (silicone elastomer, Dow Corning, Midland, MI, USA) at the bottom of the recording chamber (25 mm wide, 17 mm long, 2 mm deep). The recording chamber was mounted onto the stage of an inverted microscope (Nikon IX-70, Tokyo, Japan), and the preparation superfused with warm (35°C) Krebs solution at a constant flow rate of approximately 2 ml min\(^{-1}\).

Electrical responses of smooth muscle cells were recorded using conventional microelectrode methods. Glass capillary microelectrodes (outer diameter 1.5 mm, inner diameter 0.8 mm, Hilgenberg, Germany) filled with 3 M KCl had a tip resistance which ranged between 30 and 50 MΩ. Electrical responses, recorded via a high-input-impedance amplifier (Microelectrode Amplifier MEZ-8300, Nihon Kohden, Tokyo, Japan), were displayed on a cathode-ray oscilloscope (SS-7602, Iwatsu, Osaka, Japan) and stored on a personal computer for later analysis.
The composition of the Krebs solution was as follows (in mM): Na⁺ 137.4, K⁺ 5.9, Mg²⁺ 1.2, Ca²⁺ 2.5, HCO₃⁻ 15.5, H₂PO₄⁻ 1.2, Cl⁻ 134, glucose 11.5. The solution was aerated with O₂ containing 5% CO₂, and the pH of the solutions was 7.2–7.3.

Drugs used were acetylcholine chloride (ACh), caffeine, chelerythrine chloride, nifedipine, Nω-nitro-L-arginine (nitroarginine) and phorbol 12, 13-dibutyrate (PDBu) (purchased from Calbiochem, San Diego, CA, USA). Chelerythrine, PDBu and nifedipine were dissolved in dimethyl sulphoxide (DMSO) to make stock solutions. Other chemicals were dissolved in distilled water as a stock solution. These chemicals were diluted further with Krebs solution with the volume ratios of the dilution being over 1:1000. The dilution procedures did not alter the pH of the Krebs solution.

Experimental values were expressed as the mean value ± standard error of the mean (SEM). Statistical significance was tested using the Student’s t-test, and probabilities of less than 5% (P<0.05) were considered to be significant.

Results

Effects of PDBu on slow potentials

In the presence of nifedipine (10⁻⁶ M) and nitroarginine (10⁻⁵ M), intracellular microelectrode recordings were made from isolated circular smooth muscle bundles of the guinea-pig gastric antrum. In all preparations examined, periodic generation of slow potentials was observed, with random generation of unitary potentials during the intervals. Slow potentials had a frequency ranging from 0.6 to 3.6 cycle per min (cpm; mean, 1.6 ± 0.1 cpm, n=19) with a peak amplitude ranging from 24 to 40 mV (mean, 33 ± 1 mV, n=19). The membrane potential at its most negative value (the resting membrane potential) ranged from –52 to –78 mV (mean, –62 ± 7 mV, n=19). In each preparation, the effects of 1 mM caffeine on electrical responses were tested, and only preparations in which slow potentials and unitary potentials were abolished were accepted for the present experiments, as they were considered to contain no ICC-MY (Suzuki and Hirst, 1999).

Experiments were carried out to test the effects of three different concentrations (1, 10 and 100 nM) of PDBu on both the resting membrane potential and slow potentials in antral circular smooth muscle. PDBu produced dual effects on slow potentials, with the low concentration (1 nM) eliciting an increase in frequency of slow potentials but with the high concentration (100 nM) producing inhibition of slow potentials. At 10 nM PDBu, the frequency of slow potentials was either increased or decreased in the 5 preparations tested, with the frequency increased in 3 and decreased in 2. A typical example of the excitatory response is shown in Fig. 1A, where 1 nM PDBu increased the frequency of slow potentials with no alteration to the resting membrane potential. The effects of PDBu on unitary potentials were not clearly observed. In this preparation, the frequency of slow potentials at rest was 0.58 ± 0.16 cpm (n=8), and in the presence of 1 nM PDBu for 10–20 min, the frequency was increased to 1.21 ± 0.27 cpm (n=10, P<0.05). The amplitude of slow potentials (24.2 ± 1.3 mV, n=9) was not significantly changed in the presence of 1 nM PDBu (23.9 ± 0.7 mV, n=11; P>0.05). Addition of 100 nM PDBu resulted in an inhibition of both slow potentials and unitary potentials, with depolarization of the
membrane by about 3 mV (Fig. 1B). The inhibition by PDBu of slow potentials was reversible, but required 10–20 min for recovery (data not shown).

The effects of PDBu on the resting membrane potential and on both the amplitude and frequency of slow potentials are summarized in Fig. 2. The membrane was depolarized by 100 nM PDBu, but not by 1 and 10 nM PDBu (Fig. 2A). The amplitude of slow potentials was significantly decreased by 100 nM PDBu, but not by 1 and 10 nM PDBu (Fig. 2B). Application of 100 nM PDBu abolished slow potentials in 3 preparations and reduced the amplitude in 2 preparations. The frequency of slow potentials was variable between preparations, and therefore the change in frequency produced by PDBu was expressed as normalized value. As shown in Fig. 2C, the frequency was significantly increased by 1 nM PDBu, but significantly decreased by 100 nM PDBu. Application of 10 nM PDBu increased the frequency in 3 preparations and decreased in 2 preparations, and the averaged value of 5 preparations was not significantly different from that observed before application of PDBu. Thus, a low concentration (1 nM) of PDBu increased the frequency of slow potentials, while a high concentration (100 nM) of PDBu inhibited their frequency, with a slight depolarization of the membrane.

**Effect of chelerythrine on the actions of PDBu**

As PDBu activates protein kinase C (PKC) in many types of tissues (Nishizuka, 1984; 1992), we examined the effects of chelerythrine, a known inhibitor of PKC, on the actions of PDBu. As the above data showed that the responses of antral smooth muscle were different at low (1 nM) and high (100 nM) concentrations of PDBu, we studied the effects of chelerythrine (1–2 μM) on the actions produced by these two concentrations of PDBu. Experiments were carried out in 3 preparations, and the results on the resting membrane potential, and amplitude and frequency of slow potentials are summarized in Fig. 3 A, B and C, respectively. Chelerythrine decreased the frequency of slow potentials, with no change in the resting membrane potential and amplitude of slow potentials, which confirms our previous observations (Nakamura and Suzuki,
In the presence of chelerythrine, 1 nM PDBu did not produce any significant change in either the resting membrane potential or slow potentials. However, application of 100 nM PDBu, in the presence of chelerythrine, significantly inhibited the amplitude and frequency of slow potentials, with no significant depolarization of the membrane. Thus, chelerythrine antagonized the increase in frequency of slow potentials produced by 1 nM PDBu and the depolarization produced by 100 nM PDBu, but not the inhibition of slow potentials elicited by 100 nM PDBu.

Effect of PDBu on the ACh-induced excitatory responses

In antral circular muscle of the guinea-pig, 100 nM ACh increases the amplitude and frequency of slow potentials with no change in the resting membrane potential, with the
increased frequency of slow potentials by ACh mainly due to the activation of PKC (Nakamura and Suzuki, 2004b). Experiments were carried out to test the effects of PDBu on the excitatory actions of ACh. A typical example of the effects of PDBu on the ACh-responses is shown in Fig. 4. In the absence of PDBu, ACh (100 nM) increased the frequency and amplitude of slow potentials, with no marked change in the resting membrane potential (Fig. 4A). In the presence of 1 nM PDBu, the frequency of spontaneous slow potentials was increased, and co-application of ACh with PDBu further increased the frequency of slow potentials (Fig. 4B), but this increase was not marked compared to that in the absence of PDBu. In the presence of 100 nM PDBu, both the amplitude and frequency of slow potentials were again decreased, the former was much smaller than that in the absence of PDBu and the latter was similar to that observed in the absence of PDBu. In the presence of 100 nM PDBu, ACh produced no response (Fig. 4C). Thus, 100 nM PDBu inhibited the excitatory actions of ACh on slow potentials.

The effects of different concentrations of PDBu on the amplitude and frequency of slow potentials are summarized in Fig. 5. In the presence of ACh, the amplitude of slow potentials was inhibited by 100 nM PDBu, but not by 1 or 10 nM PDBu (Fig. 5A). Because the frequency of slow potentials varied between preparations, the value in the presence of PDBu was
expressed as relative to that measured before application of PDBu. In the presence of ACh, the frequency of slow potentials was not changed by 1 and 10 nM PDBu, but was decreased below that of the control by 100 nM PDBu (Fig. 5B). The frequency of slow potentials generated under the latter conditions was similar to that in the presence of 100 nM PDBu alone (see Fig. 2C).

**Discussion**

The present experiments revealed that in circular smooth muscle isolated from the guinea-pig stomach antrum, PDBu produced dual effects on spontaneously generated slow potentials; the frequency was increased by a low concentration (1 nM) and inhibited or abolished by a high concentration (100 nM). The latter effect was associated with a depolarization of the membrane by about 3 mV. Changes in membrane potential could modulate the amplitude and frequency of slow potentials, as depolarization has been observed to increase the frequency and decrease the amplitude (Nose *et al.*, 2000; Fukuta *et al.*, 2002). As our results have shown that a high concentration of PDBu inhibited both the amplitude and frequency of slow potentials, it would appear that the inhibition was not causally related to the depolarization of the membrane alone. Chelerythrine, a known inhibitor of PKC (Nishizuka, 1992), antagonized both the increase in frequency of slow potentials by 1 nM PDBu and the depolarization produced by 100 nM PDBu, suggesting that these two actions of PDBu are causally related to the activation of PKC.

The amplitude and frequency of slow potentials are regulated by different mechanisms, with the former being causally related to the amount of Ca²⁺ released from internal stores through activation of inositol 1,4,5-trisphosphate (IP₃) receptors, and the latter regulated by PKC (Kito *et al.*...
Indirect activation of PKC as a result of increasing the concentration of diacyl glycerol, an activator of PKC, through inhibition of diacyl glycerol lipase, could also increase the frequency of slow potentials (Suzuki et al., 2002b). If this is the case, it would be expected that activation of PKC with phorbol esters should increase the frequency of slow potentials. The present experiments have indicated that this was the case only for a concentration of 1 nM PDBu, but not at concentrations of 10 or 100 nM PDBu. Similar results have been reported for phorbol-12-myristate-13-acetate (PMA), another type of phorbol ester, in gastric muscle (Kito et al., 2002b; Lee et al., 2004; Nakamura and Suzuki, 2004b). High concentrations of PDBu inhibited the amplitude and frequency of slow potentials, as was the case for PMA in gastric muscle (Kito et al., 2002b). As the inhibition of slow potentials by a high concentration of PDBu is not antagonized by chelerythrine, causal involvement of PKC in the inhibition is not considered. Alternatively, PKC is activated by 100 nM PDBu which is much more than the level that was not able to be antagonized by micromolar concentrations of chelerythrine. In any case, the mechanism involved in the inhibition of slow potentials by high concentrations of PDBu remains unsolved.
Stimulation of muscarinic receptors by ACh increases the amplitude and frequency of slow potentials, due to an elevated release of Ca\(^{2+}\) from internal stores through activation of IP\(_3\)-receptors and the activation of PKC, respectively (Nakamura and Suzuki, 2004b). The present experiments indicate that these actions of ACh were inhibited by PDBu; a low concentration inhibited the increase in frequency and a high concentration inhibited the increase in both the amplitude and frequency. Furthermore, the excitatory actions of ACh on slow potentials were abolished in the presence of 100 nM PDBu. As the concentration of ACh tested here (equal to 100 nM) is close to the maximum that will increase the frequency of slow potentials without changing the membrane potential (Nakamura and Suzuki, 2004b), the inhibition of ACh actions by 1 nM PDBu suggests full activation of PKC during stimulation with ACh. These data could be interpreted if strong activation of PKC prevented the generation of rhythmic activity. That is, if an activation-deactivation cycle is required to produce the rhythm in gastric muscle activity, and sustained activation of PKC induces an inhibition of rhythmic activity.

The inhibition of slow potentials by 100 nM PDBu was observed with an associated inhibition of unitary potentials (Fig. 1B). As slow potentials may be formed by summated unitary potentials due to their instantaneous generation (Edwards et al., 1999), the inhibition of slow potentials may be due to the reduced generation of unitary potentials. Alternatively, strong activation of PKC could induce a disorder in the coordinated generation of unitary potentials. These unitary potentials may be generated in ICC-IM and propagated to smooth muscle cells through gap junctions, since mutant mice lacking the development of ICC-IM demonstrate neither slow potentials nor unitary potentials (Dickens et al., 2001). The conduction of electrical signals via gap junctions is not inhibited by phorbol esters (Kito et al., 2002b), suggesting that low concentrations (1 and 10 nM) of PDBu do not depolarize the membrane in both smooth muscle cells and ICC-IM. However, it remains unclear from the present experiments whether the depolarization produced by a high concentration (100 nM) of PDBu was produced in ICC-IM or smooth muscle cells, or both. In cultured ICC-MY, PKC has a negligible role in the increased frequency of spontaneous activity during muscarinic receptor stimulation (Kim et al., 2003), unlike the antral circular smooth muscle of the guinea-pig in which PKC determines the frequency of slow potentials (Nakamura and Suzuki, 2004b). It is considered that these differences may be related to the type of ICC, and that PKC may be important in frequency regulation in ICC-IM but not in ICC-MY.

It is concluded that in circular smooth muscle bundles isolated from the guinea-pig stomach antrum, PDBu produces dual effects on slow potentials; a low concentration (1 nM) increases the frequency while a high concentration (100 nM) inhibits their generation. The former may be causally related to activation of PKC, since it is antagonized by chelerythrine. The latter is not antagonized by chelerythrine, and thus the cellular mechanism of inhibition remains unclear. The excitatory actions of ACh on slow potentials are inhibited by PDBu, suggesting that sustained activation of PKC produces inhibitory effects on the rhythm of gastric muscle activity.
References


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