*Journal of Physiology* (1991), **433**, *pp.* 1–24
*With* 13 *figures*
*Printed in Great Britain*



# EFFECTS OF 2,3-BUTANEDIONE MONOXIME ON WHOLE-CELL $Ca^{2+}$ CHANNEL CURRENTS IN SINGLE CELLS OF THE GUINEA-PIG TAENIA CAECI

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(*Received 13 March 1990*)

SUMMARY

1. The inhibitory actions of cadmium ($Cd^{2+}$), nifedipine and 2,3-butanedione monoxime (BDM) on whole-cell $Ca^{2+}$ channel currents in single cells of the guinea-pig taenia caeci were investigated using a single-electrode whole-cell voltage-clamp technique.

2. Calcium channel currents were isolated using pipette solutions containing $Cs^+$, tetraethylammonium and ATP (3 mM). $Ca^{2+}$ or $Ba^{2+}$ (7·5 mM) in the bathing solution acted as the charge carrier during inward current flow. $Ca^{2+}$ channel currents in 7·5 mM-$Ba^{2+}$ ($I_{Ba}$) were recorded at potentials positive to −40 mV, were maximal near 0 mV and reversed near +60 mV. $Ca^{2+}$ channel activation showed a sigmoidal relationship with potential, which was half-maximal at −13 mV.

3. Both the inward and outward flow of current was depressed and eventually blocked by 0·3–100 μM-$Cd^{2+}$, 0·1–10 μM-nifedipine and 2–20 mM-BDM. Half-maximal blockade of $I_{Ba}$ at 0 mV was achieved with ≈ 3 μM-$Cd^{2+}$, 1 μM-nifedipine and 10 mM-BDM. Steady-state activation curves were not affected by $Cd^{2+}$ or BDM, but were shifted in the hyperpolarizing direction by nifedipine at concentrations > 1 μM.

4. Calcium channel currents in single cells and $K^+$ contractures in intact strips were both blocked in a voltage-dependent manner. Steady-state inactivation curves ($f_\infty(V)$) for $I_{Ba}$ were shifted 20 mV in the hyperpolarizing direction by 0·3 μM-nifedipine and 4 mV by 10 mM-BDM. From these shifts a dissociation binding constant to inactivated $Ca^{2+}$ channels for nifedipine was estimated as 78 nM, and for BDM, 5 mM.

5. At 10 μM $Cd^{2+}$ produced a 43 ± 6 % ($n = 3$) block of the inward current at 0 mV when $Ca^{2+}$ (7·5 mM) was the charge carrier ($I_{Ca}$), compared with the 36 ± 3 % block of $I_{Ba}$ induced by 1 μM-$Cd^{2+}$, consistent with the suggestion that $Ca^{2+}$, $Ba^{2+}$ and $Cd^{2+}$ compete for the same binding site. In contrast, nifedipine (1 μM) and BDM (10 mM) blocked $I_{Ca}$ more effectively than $I_{Ba}$.

6. Bay K 8644 (1·0 μM) increased $Ca^{2+}$ channel currents two- to fourfold at all potentials due to a shift, of ≈ 10 mV in the negative direction, of their activation curve and an equal shift in the positive direction of their inactivation curve. BDM

(5–10 mM) could antagonize the action of Bay K 8644, shifting both curves back towards their control.

7. Intracellular application of the ATP analogue, adenosine 5′*O*(3-thiotriphosphate) (ATPγS; 1 mM), which irreversibly phosphorylates cell proteins, caused substantial cell shortening but did not prevent the inhibitory action of BDM.

8. The mechanisms of action of $Cd^{2+}$, nifedipine and BDM are discussed in relation to their possible binding sites, dependent on the resting or inactivated state of the $Ca^{2+}$ channels. These results do not support the notion that BDM may be acting as a phosphatase in smooth muscle.

## INTRODUCTION

2,3-Butanedione monoxime (BDM), a member of the oxime family, has been reported to depress twitch and tetanic contractions in skeletal muscle and have a negative inotropic action in cardiac muscle. A number of sites of action have been proposed; for example concentrations of BDM < 2 mM inhibited the release of $Ca^{2+}$ from intracellular stores in mammalian skeletal muscle, but not in cardiac muscle or frog skeletal fibres (Blanchard, Alpert, Allen & Smith, 1988; Fryer, Gage, Neering, Dulhunty & Lamb 1988*a*; Fryer, Neering & Stephenson, 1988*b*; Maylie & Hui, 1988). At higher concentrations, BDM (2–10 mM) decreased the sensitivity of the contractile apparatus to $Ca^{2+}$ (Li, Sperelakis, Teneick & Solaro, 1985; Fryer *et al.* 1988*b*) and modified the number of interacting cross bridges (Blanchard *et al.* 1988). The $Ca^{2+}$ action potential in cardiac muscle and the $Ca^{2+}$ current in skeletal muscle were also reduced by these high concentrations of BDM (Bergey, Reiser, Wiggins & Freeman, 1981; Wiggins, Reiser, Fitzpatrick & Bergey, 1980; Li *et al.* 1985; Fryer *et al.* 1988*a*). Little information is available on the action of BDM in smooth muscle. The tonic contraction of the rat anococcygeus, to raised external concentrations of potassium ($K^+$) or phenylephrine (3–10 μM), was inhibited by BDM (5–30 mM) to a greater extent than the phasic component. Contractions due to the release of $Ca^{2+}$ from internal stores were only reduced slightly by BDM (20 mM) (Wendt & Lang, 1987). In single ureteral cells bathed in $Ba^{2+}$-containing solutions, BDM (5–20 mM) inhibited the action potential and $Ca^{2+}$ channel currents recorded under voltage clamp (Lang & Wendt, 1987). As oximes are mild nucleophilic agents, which can reactivate cholinesterase poisoned with organophosphorous groups (Wilson & Ginsberg, 1955; Green & Saville, 1956), it has been suggested BDM has a phosphatase-like activity and, as such, may prove a useful tool with which to study $Ca^{2+}$ channel function in smooth muscle.

Whole-cell $Ca^{2+}$ channel currents in freshly isolated single cells of the guinea-pig taenia caeci have been characterized in terms of their selectivity, saturation characteristics and kinetics of activation and inactivation, both voltage and current dependent (Ganitkevich, Shuba & Smirnov, 1986, 1987, 1988; Yamamoto, Hu & Kao, 1989*a*, *b*). The mechanisms of action of $Ca^{2+}$ entry blockers, however, have not yet been reported. In the present experiments we have compared the inhibitory actions of BDM (5–20 mM) with $Ca^{2+}$ channel blockade produced by the divalent cation $Cd^{2+}$, or by the dihydropyridine, nifedipine. We report here that all three agents reduce the inward and outward flow of current through voltage-activated $Ca^{2+}$ channels. $Cd^{2+}$ blocked by binding competitively to a site which was also

occupied by the permeating ion. Nifedipine and BDM appear to bind to another site on the $Ca^{2+}$ channel, with a greater affinity to the channel in its inactivated state. Some of these results have been communicated previously in brief (Lang & Paul, 1989; Paul, Wendt & Lang, 1989).

## METHODS

### *Cell dispersal*

Single smooth muscle cells were isolated from 1 cm lengths of taenia caeci of the guinea-pig killed previously with a blow to the head. Strips were bathed in a physiological salt solution (at 37 °C) with added collagenase (0·6 mg/ml), trypsin inhibitor (0·5 mg/ml), bovine serum albumin (2 mg/ml) and $Ca^{2+}$ (30 $\mu$M) for 60 min and then transferred to an enzyme-free saline; single cells were dispersed by gentle agitation using a wide-bore pipette. $Ca^{2+}$ (0·4 mM) was added to the cells in suspension, which were then plated on glass cover-slips and stored at 4 °C. Cover-slips were placed on an inverted microscope. Single cells were viewed under bright-field illumination on a TV monitor.

### *Whole-cell current recording*

Low-resistance patch pipettes (2–7 MΩ) were gently pushed against the cell membrane. A high-resistance (> 8 GΩ) seal was formed by applying a slight suction. Sharp suction and/or voltage were applied to rupture the underlying membrane. Whole-cell currents were recorded (at room temperature; 22–25 °C) using standard patch-clamp techniques with an Axopatch 1B patch-clamp apparatus (Axon Instruments) (Lang, 1989, 1990).

### *Intact tissue mechanical measurements*

Strips of taenia caeci were dissected and cut into 1·5 cm lengths. Tissues were mounted isometrically in a 20 ml organ bath and equilibrated for at least 1 h in salt solution containing 1·5 mM-$Ca^{2+}$. Strips were placed under an initial load of 10 mN and, following development of spontaneous contractions, a test contraction was induced by the addition of 40 mM-KCl. The tissues were then relaxed in a nominally $Ca^{2+}$-free saline. After relaxation, the tissues in $Ca^{2+}$-free saline were shown to be unresponsive to subsequent depolarization by 40 mM-KCl. At this point the experimental protocol described in Fig. 11 was initiated.

The percentage inhibition of $K^+$ contractions of the intact tissue was quantified by measuring the area under the isometric force myogram for the initial 2 min of contracture. The area of the isometric myogram in the presence of these blockers was expressed as a percentage of the area of a control contraction for each strip.

### *Solutions*

Cells were bathed and initially impaled in a salt solution containing (mM): NaCl, 126; KCl, 5·9; sodium HEPES, 6; glucose, 11; $MgCl_2$, 1·2; and $CaCl_2$, 1·5; adjusted to pH 7·4 with 5 M-NaOH. Currents through $Ca^{2+}$ channels were recorded in a similar saline in which $K^+$ was substituted by $Cs^+$, tetraethylammonium (TEA; 5 mM) added, and $Ca^{2+}$ or $Ba^{2+}$ was 7·5 mM; pH was adjusted to 7·4 with 5 M-TEAOH. The pipette solution contained (mM): CsCl, 130; sodium HEPES, 6–10; ATP, 3; EGTA, 3; $MgCl_2$, 2·9; glucose, 11; TEA, 5; and buffered to pH 7·4 with TEAOH (Lang, 1989, 1990).

### *Data analysis*

Electrophysiological data were recorded on a video cassette recorder using a digital data recorder (List VR-10) and then analysed after digitization with a Labmaster analog-to-digital interface using an Arrow-AT personal computer and p-CLAMP software (Axon Instruments).

*Steady-state activation and inactivation curves.* The potential dependence of the activation of $I_{Ba}$ was estimated by assuming that the maximal amplitude of $I_{Ba}$ at any potential $V$ (V) was made up of its driving force ($V-E_{REV}$) and peak conductance ($g(V)$, where $E_{REV}$ is the reversal potential of $I_{Ba}$ obtained experimentally, such that

$$I_{peak}(V) = g(V)\,(V-E_{REV}).$$

$g(V)$ at any potential can be expressed as a fraction of the maximally available conductance ($g^*$) such that

$$g(V) = g^* d_\infty(V),$$

where $d_\infty(V)$ is the steady-state activation parameter (Hodgkin & Huxley, 1952; Klöckner & Isenberg, 1985; Lang, 1990). It follows that

$$I_{peak}(V) = g^* d_\infty(V)(V - E_{REV}),$$

where $d_\infty(V)$ is described by a Boltzmann distribution of

$$d_\infty(V) = \{1 + \exp[(V_{0.5} - V)/k]\}^{-1},$$

and $V_{0.5}$ is the potential of half-maximal activation, $k$ is a slope factor. In cells where it was not possible to estimate and subtract the background membrane conductance (in 100 $\mu$M-$Cd^{2+}$) plots were fitted to the equation

$$d_\infty(V) = (1 - R)\{1 + \exp[(V_{0.5} - V)/k]\}^{-1} + R,$$

where $R$ is an estimate of the resting conductance at negative potentials.

The sigmoidal, steady-state inactivation (or availability) curve ($f_\infty(V)$) was fitted through the data, by a least-squares fit, with

$$f_\infty(V) = \{1 + \exp[(V_{0.5} - V)/k]\}^{-1},$$

where $V_{0.5}$ is the potential of half-maximal availability, $k$ is a slope factor. Again currents in response to the test depolarization, to 0 mV, were first corrected by subtracting the background membrane currents recorded in 100 $\mu$M-$Cd^{2+}$.

## RESULTS

### *Morphology and electrical characteristics of single cells*

The enzymatic dispersal yielded cells of varying lengths; many were elongated cylinders 100–300 $\mu$m in length and 5–10 $\mu$m in diameter (Yamamoto *et al.* 1989*a*). Cells < 200 $\mu$m (e.g. 134·6 ± 6·8 $\mu$m ($N = 19$); mean ± standard error of the mean) and which did not contract upon exposure to $Ca^{2+}$-containing saline (1·5 mM-$Ca^{2+}$) were generally used in the present experiments.

Table 1 summarizes the basic electrical properties of eleven single cells recorded with $Cs^+$-filled patch pipettes as revealed under voltage clamp when the membrane potential was stepped from −80 to −90 mV for 10 ms. The averaged input resistance ($r_{input}$) of these eleven cells was 1·53 ± 0·1 GΩ. The averaged whole-cell capacitance of 53 ± 3 pF was measured by dividing the time integral of the capacitive artifact (pA ms) by the amplitude of the hyperpolarization (10 mV), after correcting previously for the resting, 'leak' conductance. Dividing this capacity by an assumed specific capacitance of 1 $\mu$F/cm$^2$ gives an estimate of the surface area of an average cell as $0{\cdot}53 \pm 0{\cdot}03 \times 10^{-4}$ cm$^2$. Multiplying the averaged membrane resistance by the surface area also gives a specific input resistance ($R_m$) of these $Cs^+$-filled cells of 81 kΩ cm$^2$. The decay of the capacitive current was well fitted by a single exponential with an averaged time constant of 0·56 ± 0·05 ms. The effective series resistance can be calculated by dividing this time constant by the membrane capacity. With an averaged effective series resistance of 10·4 ± 0·5 MΩ and maximal current flow in these cells about 0·5 nA, errors in the recorded values of membrane potential would be less than 5 mV.

*Voltage-activated $Ca^{2+}$ channel currents*

Time-dependent membrane currents ($I_{Ba}$) were recorded in most cells bathed in 7·5 mM-$Ba^{2+}$ at potentials positive to −40 mV, when triggered by depolarizing command pulses (0·2–1 s duration at a frequency of 0·05–0·03 Hz) from a holding potential of −80 mV. These currents rose to a peak amplitude within 10 ms and then

TABLE 1. Electrical properties of single cells of the taenia caeci

| | Input resistance (MΩ) | Cell capacitance (pF) | Cell area ($\times 10^{-4}$ cm$^2$) | Capacitative time constant (ms) | Series resistance (mΩ) |
|---|---|---|---|---|---|
| Cell 1 | 991 | 68·14 | 0·68 | 0·94 | 13·8 |
| Cell 2 | 1868 | 58·37 | 0·58 | 0·73 | 12·5 |
| Cell 3 | 1296 | 46·12 | 0·46 | 0·47 | 10·2 |
| Cell 4 | 1706 | 63·74 | 0·64 | 0·60 | 9·4 |
| Cell 5 | 1650 | 48·73 | 0·49 | 0·47 | 9·6 |
| Cell 6 | 1257 | 58·12 | 0·50 | 0·58 | 10·0 |
| Cell 7 | 1657 | 45·96 | 0·40 | 0·54 | 11·75 |
| Cell 8 | 2244 | 43·75 | 0·44 | 0·40 | 9·1 |
| Cell 9 | 1292 | 68·92 | 0·69 | 0·64 | 9·3 |
| Cell 10 | 1658 | 43·19 | 0·43 | 0·48 | 11·1 |
| Cell 11 | 1235 | 38·85 | 0·39 | 0·29 | 7·5 |
| Average | 1532 | 53·08 | 0·53 | 0·56 | 10·40 |
| ±S.E.M. | 102 | 3·08 | 0·05 | 0·05 | 0·5 |
| (*n*) | (11) | (11) | (11) | (11) | (11) |

slowly inactivated such that there was still a substantial current after 400 ms. $I_{Ba}$ in most cells increased in amplitude over the first 5–10 min with little change in its time course (Fig. 1*A* and *B*) (Langton, Burke & Sanders, 1989). This was then followed by a time-dependent run-down in the amplitude of $I_{Ba}$ (Fig. 1*A*). The rate of run-down was extremely variable ranging from 1 to 10% of the initial amplitude per minute (Lang, 1989). In some cells the initial amplitude of $I_{Ba}$ upon impalement was particularly large, 400–500 pA (Yamamoto *et al.* 1989*a*); these cells generally showed an initial rapid run-down for the first few minutes to approximately 50% of their initial amplitude which was then followed by a slower, more consistent phase.

In Fig. 2*A* membrane currents every 20 mV between −60 and +100 mV have been superimposed. Inward currents were maximal near 0 mV; at more positive potentials these currents changed progressively from a decaying inward current to a decaying outward current. Near +60 mV no time-dependent currents were recorded. The early peak current ($I_{(peak)}$; ●) and the current at the end of these 400 ms depolarizations ($I_{(400)}$; ○) in 7·5 mM-$Ba^{2+}$ have been averaged in six cells and plotted against potential in Fig. 2*B*. These plots demonstrate the current–voltage (*I–V*) relationship of these membrane currents activated by potential. These currents, however, are the product of the conductance activated at each potential and their driving force. To estimate better the potential range over which these currents are activated, normalized steady-state activation curves for $I_{(peak)}$ and $I_{(400)}$ have been plotted using a value of $E_{REV}$ of +63 mV, the potential at which the two

curves in Fig. 2*B* intersect and where no active membrane currents were recorded (Fig. 2*A*). $I_{(peak)}(V)/(V\text{-}63)$ and $I_{(400)}(V)/(V\text{-}63)$ at potentials between −80 and +40 mV were therefore expressed as a fraction of their maximally obtained conductance and plotted against potential in Fig. 2*C*. It can be seen that threshold

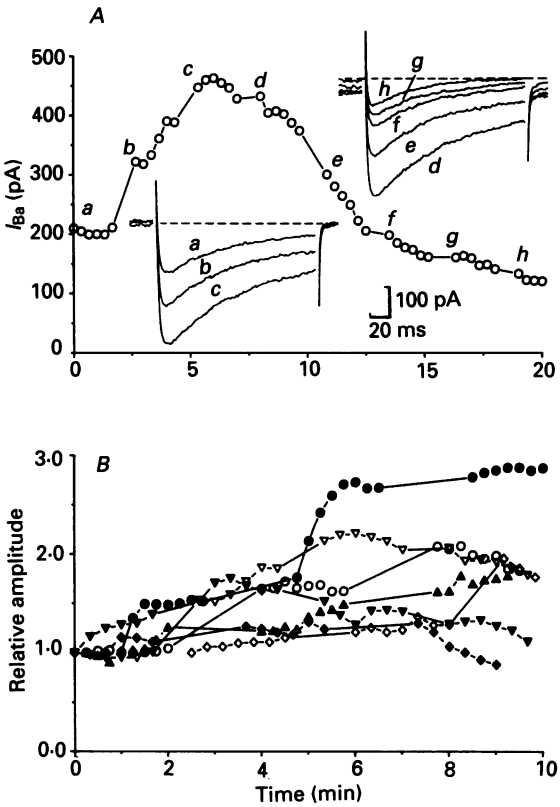


Fig. 1. Effect of perfusion time on the amplitude of whole-cell $Ca^{2+}$ channel currents. *A*, plot of the initial increase and subsequent decrease of the peak amplitude of $I_{Ba}$, at 0 mV, plotted against time; holding potential −80 mV, cell bathed in 7·5 mM-$Ba^{2+}$. *B*, initial increase of the normalized peak amplitude of $I_{Ba}$ in seven cells plotted against time.

for both currents was near −40 mV and that both were maximal near 0 mV. Sigmoidal curves have been fitted, using a least-squares algorithm, to these plots such that $V_{0.5}$ for $I_{(peak)}$ and $I_{(400)}$ was −13·1 and −16·2 mV respectively (while $k = 6{\cdot}38$ and 5·73; $R = 0{\cdot}039$ and 0·112 respectively). Inward currents at potentials negative of the null potential and the outward currents at potentials more positive were both blocked upon $Ca^{2+}$ entry blockade (see below); these currents are therefore thought to arise from the inward flow of $Ba^{2+}$ and the outward flow of $Cs^{+}$ ions through voltage-activated $Ca^{2+}$ channels (Lee & Tsien, 1984; Tsien, Hess, McCleskey & Rosenberg, 1987). The maximal membrane conductance of these cells was 3·1 nS in 7·5 mM-$Ba^{2+}$. Assuming a surface area of about $0{\cdot}5 \times 10^{-4}$ $cm^2$, as estimated from

the cell capacitance (Table 1; Yamamoto *et al.* 1989*a*) this gives a maximum membrane conductance ($g^*_{Ba}$) of 6·2 mS/cm².

*One or two types of $Ca^{2+}$ channel current*

Two types of whole-cell $Ca^{2+}$ current have been shown to co-exist in a variety of visceral and vascular smooth muscles (Bean, Sturek, Puga & Hermsmeyer, 1986;

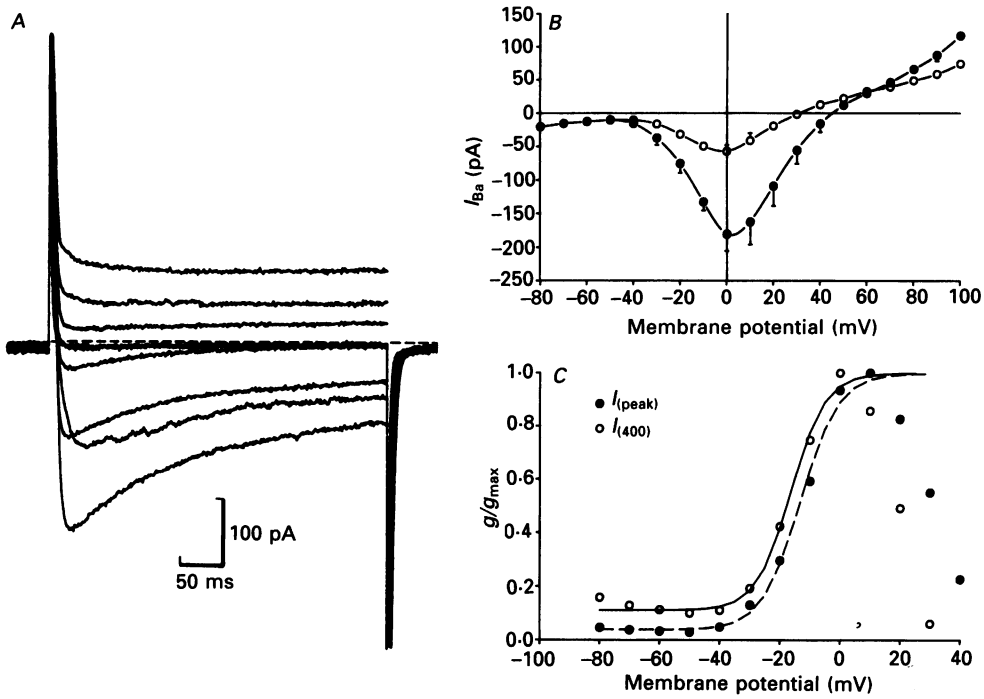


Fig. 2. *I–V* relationship of $I_{Ba}$. *A*, current responses to depolarizing steps (400 ms duration) of potential which increased 20 mV successively; holding potential −80 mV. Threshold for $I_{Ba}$ was positive to −40 mV, $I_{Ba}$ was maximal near 0 mV and had a reversal potential near +60 mV. *B*, plot of the *I–V* relationship for $I_{Ba}$ averaged from six cells. $I_{(peak)}$ represents the peak amplitude of $I_{Ba}$, while $I_{(400)}$ represents the amplitude of $I_{Ba}$ at the end of these 400 ms depolarizations. *C*, voltage-dependent activation of $I_{Ba}$. Relative conductances of $I_{(peak)}$ and $I_{(400)}$ were calculated and plotted against potential using an $E_{REV}$ of +63 mV. Smooth curves through normalized points are Boltzmann distributions of the form

$$d_\infty(V) = (1-R)\{1+\exp[(V_{0.5}-V)/k]\}^{-1}+R,$$

where $V_{0.5}$ is the potential of half-maximal activation, $k$ is a slope factor and $R$ is the resting conductance. $V_{0.5}$, $k$ and $R$ were −13·1, 3·8 and 0·04 for $I_{(peak)}$; −16·2, 5·7 and 0·11 for $I_{(400)}$.

Hirst, Silverberg & van Helden, 1986; Loirand, Pacaud, Mironneau & Mironneau, 1986; Benham, Hess & Tsien, 1987; Yatani, Seidel, Allen & Brown, 1987; Aaronson, Bolton, Lang & MacKenzie, 1988; Yoshino, Someya, Nishio & Yabu, 1988) but not in all (Nakazawa, Saito & Matsuki, 1988; Honoré, Amédée, Martin, Dacquet,

Mironneau & Mironneau, 1989; Katzka & Morad, 1989; Lang, 1990). They are thought to arise from two separate populations of $Ca^{2+}$ channels which can be distinguished in terms of their conductance, kinetics, voltage dependencies and sensitivities to pharmacological agents. One $I_{Ca}$ is transient in nature and is thought

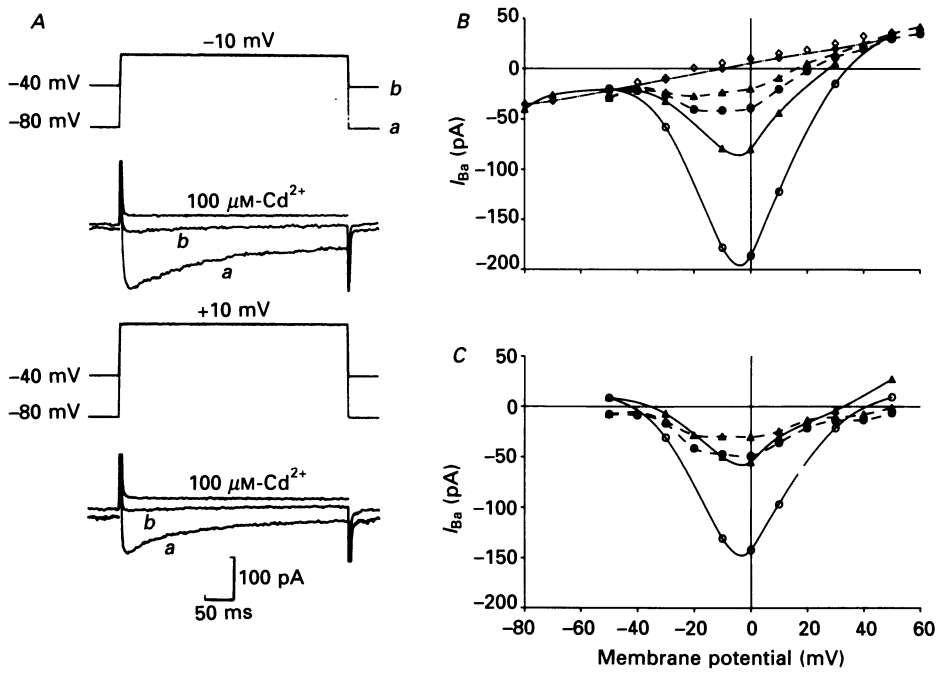


Fig. 3. Influence of holding potential on $I_{Ba}$. Superimposed membrane currents in 7·5 mM-$Ba^{2+}$ at −10 (*A*, upper panel) and +10 mV (*A*, lower panel) elicited from holding potentials of −80 (*a*) and −40 (*b*) mV in the absence and presence of 100 μM-$Cd^{2+}$. *B*, plot of *I–V* relationship of $I_{Ba}$ triggered from −80 mV ($I_{(peak)}$, ○; $I_{(400)}$, △) and −40 mV ($I_{(peak)}$, ●; $I_{(400)}$, ▲) in the absence and presence of 100 μM-$Cd^{2+}$ ($I_{(400)}$, ◇). *C*, currents recorded in $Cd^{2+}$ were subtracted from those recorded at −40 and −80 mV. The $Cd^{2+}$-sensitive currents at −40 mV (●, ▲) and the $Cd^{2+}$-sensitive currents at −80 mV minus those at −40 mV (○, △) are plotted against potential.

to arise from a population of $Ca^{2+}$ channels of a small conductance, which activate and inactivate rapidly at negative potentials (< −40 mV). The other $Ca^{2+}$ current inactivates more slowly upon depolarization and is thought to arise from a population of $Ca^{2+}$ channels which have a larger conductance, an activation range dependent on the divalent cation concentration and a greater sensitivity to the actions of dihydropyridine drugs (Bean *et al.* 1986; Hirst *et al.* 1986; Loirand *et al.* 1986; Benham *et al.* 1987; Yatani *et al.* 1987; Aaronson *et al.* 1988).

Single-channel recordings from cells of the guinea-pig taenia caeci have demonstrated up to three subtypes of $Ca^{2+}$ channel, distinguished by their unit conductance in high $Ba^{2+}$ concentrations and their time course of activation and inactivation. Besides the two populations of channels described above, a third

population of $Ca^{2+}$ of intermediate conductance which are slowly activating and non-inactivating was recently reported (Yoshino *et al.* 1988; Yabu, Yoshino, Someya, Usuki, Obara & Tostuka, 1989; Yoshino, Someya, Nishio, Yazawa, Usuki & Yabu, 1989). We have attempted to distinguish these channel subtypes by voltage alone

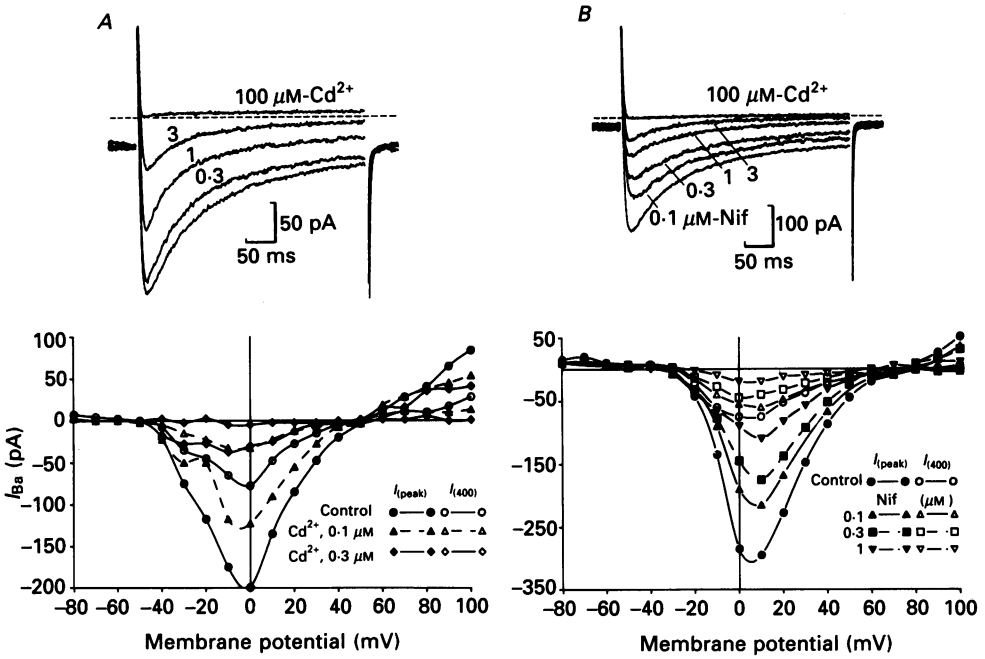


Fig. 4. Effects of cadmium ($Cd^{2+}$, 0·1–100 μM; *A*) and nifedipine (Nif, 0·1–3·0 μM; *B*) on $I_{Ba}$ and its *I–V* relationship. Top panels, $Cd^{2+}$ and nifedipine dose dependently reduced the amplitude of $I_{Ba}$ at 0 mV; holding potential −80 mV. Lower panels, plot of the *I–V* relationship of $I_{(peak)}$ and $I_{(400)}$ for $I_{Ba}$ in control and indicated concentrations of $Cd^{2+}$ and nifedipine. Note that both the inward and outward flow of current through these smooth muscle $Ca^{2+}$ channels are blocked, as is evident by the reduction of the currents at potentials negative and positive to the $I_{Ba}$ reversal potential.

using whole-cell voltage-clamp protocols, similar to that designed by Bean (1985) to separate the two $I_{Ca}$ types in heart cells. Cells, bathed in 7·5 mM-$Ba^{2+}$ saline, were held at −80 and −40 mV for 1–2 min before test depolarizations to various potentials were applied. Illustrated in Fig. 3*A* are the currents recorded at −10 and +10 mV when elicited from −40 and −80 mV in the absence and presence of 100 μM-$Cd^{2+}$. The $Cd^{2+}$-sensitive $I_{Ba}$ showed a marked sensitivity to potential; decreasing as the holding potential was more positive. The *I–V* relationship of $I_{Ba}$ at −80 and −40 mV has been plotted in Fig. 3*B*. When the holding potential was −40 mV, $I_{(peak)}$ and $I_{(400)}$ were smaller at every membrane potential. Threshold and the potential at which $I_{(peak)}$ and $I_{(400)}$ were maximal (−40 and 0 mV respectively) were little affected by the holding potential. The *I–V* relationship of the $Ca^{2+}$ channels that can be opened only with a holding potential of −80 mV was obtained by subtracting the $Cd^{2+}$-sensitive $I_{(peak)}$ and $I_{(400)}$ at −40 mV from the $Cd^{2+}$-sensitive currents at −80 mV (Fig. 3*C*; ○, △). This difference current had a similar *I–V* relationship to those obtained at −80 or −40 mV (Lang, 1990). Therefore, voltage

alone could not distinguish at the whole-cell current level (in 7·5 mM-$Ba^{2+}$) the three populations of $Ca^{2+}$ channels suggested to be present in the taenia caeci, presumably due to their overlapping potential ranges of activation (Yoshino *et al.* 1989).

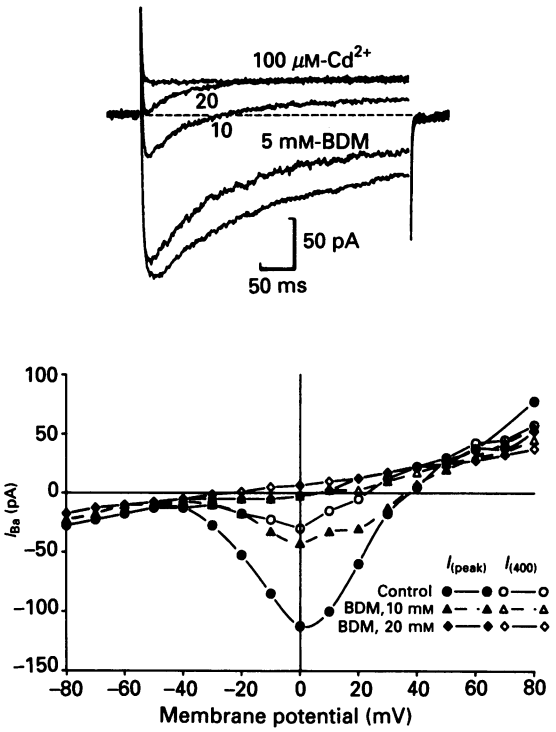


Fig. 5. BDM (5–20 mM) also blocks both the inward and outward flow of current through these smooth muscle $Ca^{2+}$ channels. Conditions as in Fig. 4.

*Effects of $Cd^{2+}$, nifedipine and BDM*

Figures 4 and 5 illustrate the effects of $Cd^{2+}$, nifedipine and BDM, at the concentrations indicated, on the amplitude of $I_{Ba}$ at 0 mV from a holding potential of −80 mV. $Cd^{2+}$ and nifedipine reduced $I_{Ba}$ at concentrations > 0·1 μM. Complete blockade of $I_{Ba}$ was usually achieved with 100 μM-$Cd^{2+}$; this concentration of $Cd^{2+}$ was therefore used in all experiments to indicate background membrane and capacitive currents. Where possible these background membrane currents were subtracted from *I–V* plots before display (Fig. 4*A* and *B*). BDM reduced $I_{Ba}$ at concentrations > 2 mM (Fig. 5). All three blockers dose dependently reduced $I_{(peak)}$ and $I_{(400)}$ at potentials negative and positive to $E_{REV}$ (Figs 4 and 5). Half-maximal blockade of the $I_{(peak)}$ of $I_{Ba}$, at 0 mV, was achieved with 1 μM-nifedipine (49±9% ($n = 5$) block) and 10 mM-BDM (51±5 ($n = 10$)); whereas 1 μM-$Cd^{2+}$ produced a 36±4% ($n = 3$) inhibition.

To determine accurately the effect of $Cd^{2+}$, nifedipine and BDM on $Ca^{2+}$ channel activation, peak amplitudes of $I_{Ba}$ in the various blocker concentrations were calculated in terms of absolute and normalized conductance and plotted against potential. $Cd^{2+}$ (0·3–1 μM; Fig. 6*A*), nifedipine (0·1–0·3 μM; Fig. 6*B*) and BDM (5–10 mM; Fig. 7) reduced $Ca^{2+}$ channel conductance without affecting the potential

range of activation. Nifedipine at concentrations > 1·0 μM, on the other hand, induced a shift in the voltage dependence of activation in the hyperpolarizing direction (Fig. 6*B*). $V_{0.5}$ of the normalized steady-state activation curve was −7·4 mV in control saline and −6·3 mV when in 0·1 or 0·3 μM-nifedipine; but −8·8 and

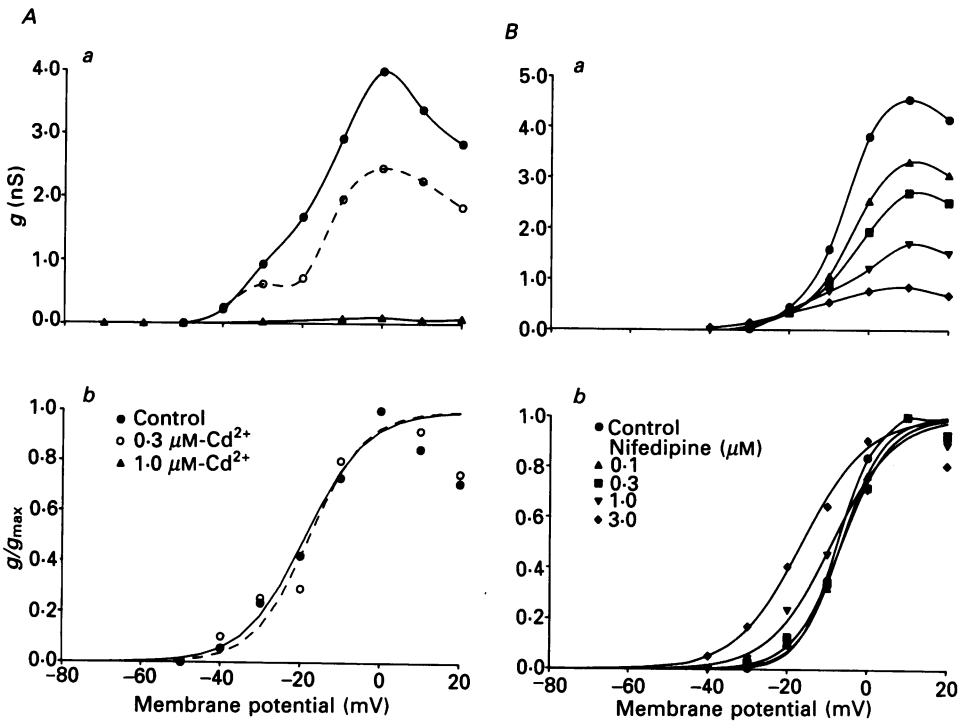


Fig. 6. Effects of 0·3–1 μM-$Cd^{2+}$ (*A*) and 0·1–3 μM-nifedipine (*B*) on voltage-dependent activation on $I_{Ba}$. Absolute (*Aa*, *Ba*) and relative (*Ab*, *Bb*) conductance of the $Ca^{2+}$ channel currents were calculated in the absence and presence of the blockers at the concentrations indicated and plotted against potential. Lines through relative data (lower panels) were Boltzmann distributions, fitted by least squares, of the form

$$d_\infty(V) = \{1 + \exp[(V_{0.5} - V)/k]\}^{-1},$$

where $V_{0.5}$ is the potential of half-maximal activation, $k$ is a slope factor. The values of $V_{0.5}$ in control and 0·3 μM-$Cd^{2+}$ were −18·7 and −17·66 (*Aa*). $V_{0.5}$ for control, 0·1, 0·3, 1 and 3 μM-nifedipine were −7·4, −6·3, −6·3, −8·8 and −16·3 mV respectively (*Bb*).

−16·3 mV when nifedipine was raised to 1 and 3 μM (Fig. 6*Bb*). Similar effects of nifedipine were seen in three other cells. Nifedipine and CV-4093, another dihydropyridine, increased inward currents at negative potentials in single cells of the ear and pulmonary artery of the rabbit (Okabe, Terada, Kitamura & Kuriyama, 1987; Aaronson *et al.* 1988).

### *Effects of $Cd^{2+}$, nifedipine and BDM on $I_{Ba}$ and $I_{Ca}$*

Inward current flow through $Ca^{2+}$ channels was generally larger when an equimolar concentration of $Ba^{2+}$ currents was substituted for $Ca^{2+}$ (Tsien *et al.* 1987). It is thought that $Ba^{2+}$ binds less strongly to a site within the channel (metal co-

ordination site) which results in a greater $Ba^{2+}$ mobility through the channel. The actions of most $Ca^{2+}$ channel blockers can usually be antagonized by raising the extracellular concentration of $Ca^{2+}$. They may not, however, be acting at the same site within the channel. We have examined this possibility by comparing the action of the three blockers, when $Ca^{2+}$ or $Ba^{2+}$ acted as the permeant ion.

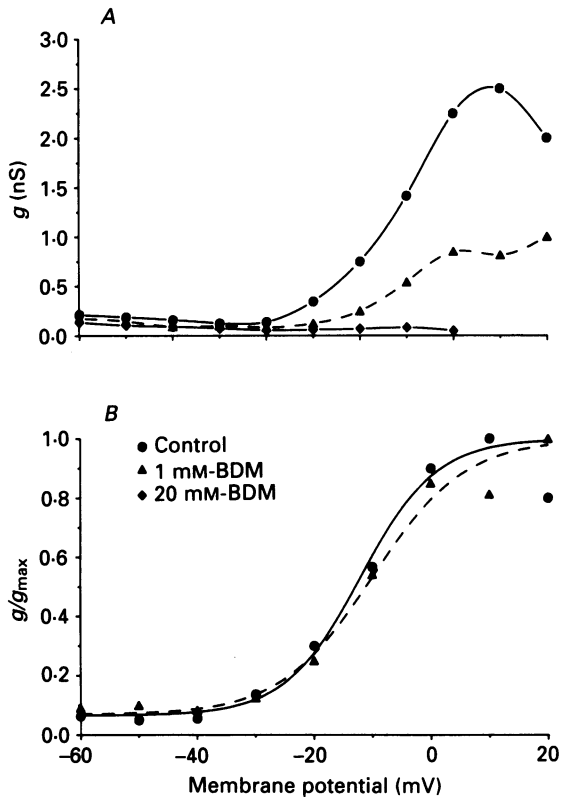


Fig. 7. Effects of BDM (10–20 mM) on voltage-dependent activation of $I_{Ba}$. Absolute (*A*) and relative (*B*) conductance have been plotted against potential as in Fig. 6. In this cell the resting conductance (*R*) was not subtracted previously. Therefore the data were fitted with a modified Boltzmann distribution of

$$d_\infty(V) = (1-R)\{1+\exp[(V_{0.5}-V)/k]\}^{-1}+R,$$

such that $V_{0.5}$ and $R$ were $-12$ mV and 0·07 respectively for control; $-9.9$ mV and 0·07 for 10 mM-BDM.

In the cell illustrated in Fig. 8*A*, $I_{Ca}$ (7·5 mM-$Ca^{2+}$) was approximately one-third of the amplitude of $I_{Ba}$ (7·5 mM-$Ba^{2+}$); the averaged peak amplitude of $I_{Ca}$ in sixteen cells, however, was $51\pm5\%$ of $I_{Ba}$. In this cell 1 $\mu$M-$Cd^{2+}$ produced an approximate 50% blockade of $I_{Ba}$ but only slightly reduced $I_{Ca}$. On the other hand, 10 $\mu$M-$Cd^{2+}$ was required to produce an approximate 50% block of $I_{Ca}$. Nifedipine (0·3 $\mu$M) and BDM (10 mM) showed the opposite behaviour; blocking $I_{Ca}$ more effectively than $I_{Ba}$ (Fig. 8*B*).

These results are consistent with $Cd^{2+}$, $Ca^{2+}$ and $Ba^{2+}$ binding to the same site, $Ba^{2+}$ with a lesser affinity would be more affected by the strongly binding $Cd^{2+}$. The greater blockade of $I_{Ca}$ by nifedipine and BDM cannot be explained by a simple

competition at this site of $Cd^{2+}$ action, but must involve a separate site at which the binding of $Ca^{2+}$ or $Ba^{2+}$ can affect the action of these two blockers (Lee & Tsien, 1984; Tsien *et al.* 1987).

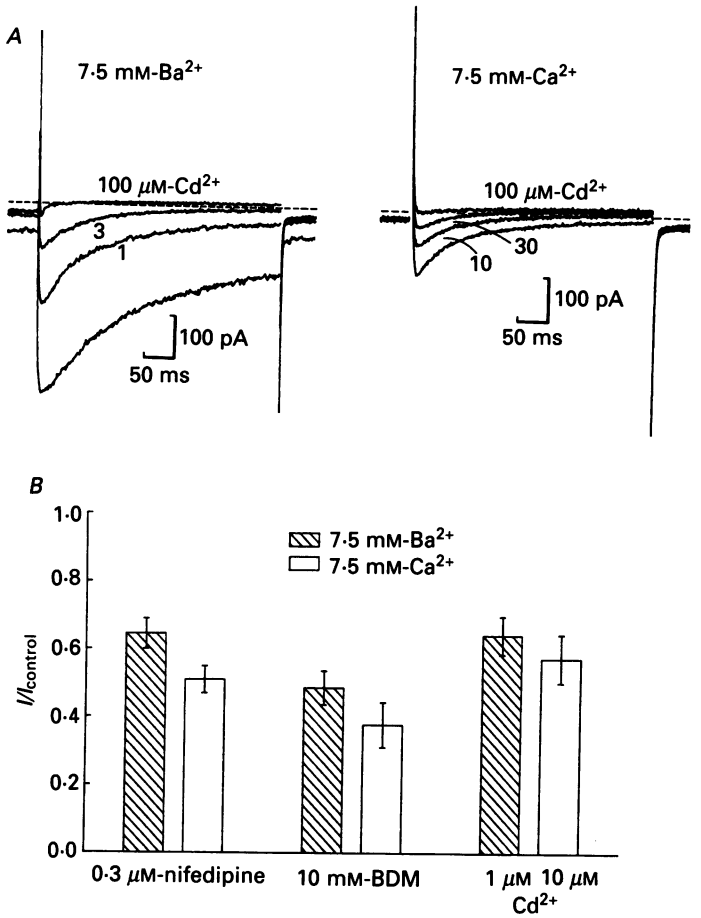


Fig. 8. A comparison of the effects of $Cd^{2+}$, nifedipine and BDM on $I_{Ba}$ (7·5 mM-$Ba^{2+}$) and $I_{Ca}$ (7·5 mM-$Ca^{2+}$) at 0 mV, from a holding potential of −80 mV. *A*, a tenfold larger concentration of $Cd^{2+}$ was required to produce an equivalent block of $I_{Ca}$ to that seen with $I_{Ba}$. *B*, 0·3 μM-nifedipine and 10 mM-BDM, however, were more effective at blocking $I_{Ca}$ than $I_{Ba}$.

*Initial, conditioned or tonic block of* $I_{Ba}$

Blockade of $I_{Ba}$ by $Cd^{2+}$ and BDM was invariably associated with an acceleration of current decay; blockade by nifedipine, however, was not (Figs 4 and 5). An acceleration of the inactivation of the $Ca^{2+}$ channel current in the presence of a channel blocker has been postulated to reflect (i) a preferential block of a distinct class of slowly inactivating $Ca^{2+}$ channels (Bean *et al.* 1986) or (ii) a preferential blockade of open or inactivated $Ca^{2+}$ channels (Lee & Tsien, 1983). Blockage of open $Ca^{2+}$ channels can be tested using stimulation protocols similar to those devised for studying the blockade of $Na^{+}$ channels by local anaesthetics (Hille, 1977) or the action of $Ca^{2+}$ antagonists on $Ca^{2+}$ channels in single cardiac cells (Lee & Tsien, 1983).

Four to eight $Ba^{2+}$ currents were recorded in the absence of blockers with test depolarizations (400 ms in duration) to 0 mV every 20 or 30 s. Cells were then rested for 2–3 min and then stimulated again with a similar number of test depolarizations. This procedure was repeated until the rate of $I_{Ba}$ run-down was established. BDM (5–10 mM), nifedipine (1 $\mu$M) or $Cd^{2+}$ (3 $\mu$M) were then added at the beginning of a rest period.

$I_{(peak)}$ and $I_{(400)}$ of the first $I_{Ba}$ in the next train of depolarizations were dose dependently reduced by all three blockers, but no further during the train of stimulation (data not shown) (Lee & Tsien, 1983; Hering, Beech, Bolton & Lim, 1988). Small progressive decreases in $I_{Ba}$ in the presence of blocking drugs were sometimes seen, but were not relieved by a subsequent rest period. This was explained as $Ca^{2+}$ channel run-down. Conditioned block is usually characterized by a near normal amplitude of $I_{Ba}$ after a rest period, followed by a progressive reduction in $I_{Ba}$ amplitude during repetitive depolarization. The pronounced block of $I_{Ba}$ after a rest period and the lack of any conditioned block in the present experiments, even though $I_{Ba}$ decayed more rapidly, suggests that $Cd^{2+}$, nifedipine or BDM are not rapidly blocking open channels (Hering *et al.* 1988).

*Voltage-dependent action of BDM, nifedipine and $Cd^{2+}$*

The blockade of whole-cell $Ca^{2+}$ channel currents in smooth muscle by dihydropyridines is known to be strongly voltage dependent. $Ca^{2+}$ channel currents obtained from positive holding potentials (−40 to −30 mV) are more readily blocked by a number of dihydropyridines, at concentrations 10–100 times less than that required to block currents elicited from more negative holding potentials (−80 to −60 mV) (Bean *et al.* 1986; Yatani *et al.* 1987; Nakazawa *et al.* 1988).

In Fig. 9, $I_{Ba}$ recorded at 0 mV, from holding potentials of −80 and −40 (or −30 mV), have been superimposed in the absence and presence of BDM (10 mM), nifedipine (0·3 $\mu$M) and $Cd^{2+}$ (1 $\mu$M). $I_{(peak)}$ of $I_{Ba}$ from −40 mV was generally about 60% of that elicited from −80 mV in control solutions (Fig. 9, left column; see below). The action of 0·3 $\mu$M-nifedipine was markedly voltage dependent. $I_{(peak)}$ was reduced by 40% when $I_{Ba}$ was elicited from −80 mV, but almost completely when $I_{Ba}$ was triggered from −40 mV. $Cd^{2+}$ and BDM also showed a slight voltage dependence in their blockade of $I_{Ba}$, such that $I_{(peak)}$ was relatively smaller when $I_{Ba}$ was elicited from positive potentials (Fig. 9, right column).

This voltage dependence was examined using a double-pulse protocol. A test pulse (duration 200 ms every 30 s) to 0 mV from the holding potential of −80 mV was preceded by a 1 s conditioning pulse of various amplitudes. To remove the effects of $Ca^{2+}$ channel run-down, pairs of stimulations were applied; the first without (current 2 in Fig. 10*A*), the second with the conditioning pulse (current 1 in Fig. 10*A*). Currents in control saline recorded in response to test pulses after a conditioning pulse were expressed as a fraction of its accompanying conditioning-free test $I_{Ba}$. These fractions were then normalized as a fraction of the maximum value obtained and plotted against potential (Fig. 10*B*). Test $I_{Ba}$'s in nifedipine (0·3 $\mu$M) or BDM (10 mM) were either expressed as a fraction of the averaged conditioning-free $I_{Ba}$ in control saline (Fig. 10*Ba* and *c*), or normalized as above (Fig. 10*Bb* and *d*). In control saline the amplitude of the conditioning pulse to reduce $I_{Ba}$ to half its amplitude ($V_{0.5}$) was $-36{\cdot}0 \pm 1{\cdot}5$ ($n = 3$) mV. Application of nifedipine (0·3 $\mu$M) or BDM (10 mM)

reduced the relative amplitude of $I_{Ba}$ at all conditioning potentials (Fig. 10*Ba* and *c*). $V_{0.5}$ was shifted from −32·4 to −52·1 mV in nifedipine (0·3 μM) (Fig. 10*Bb*), and −37·3 to −41·1 mV in BDM (10 mM) (Fig. 10*Bd*).

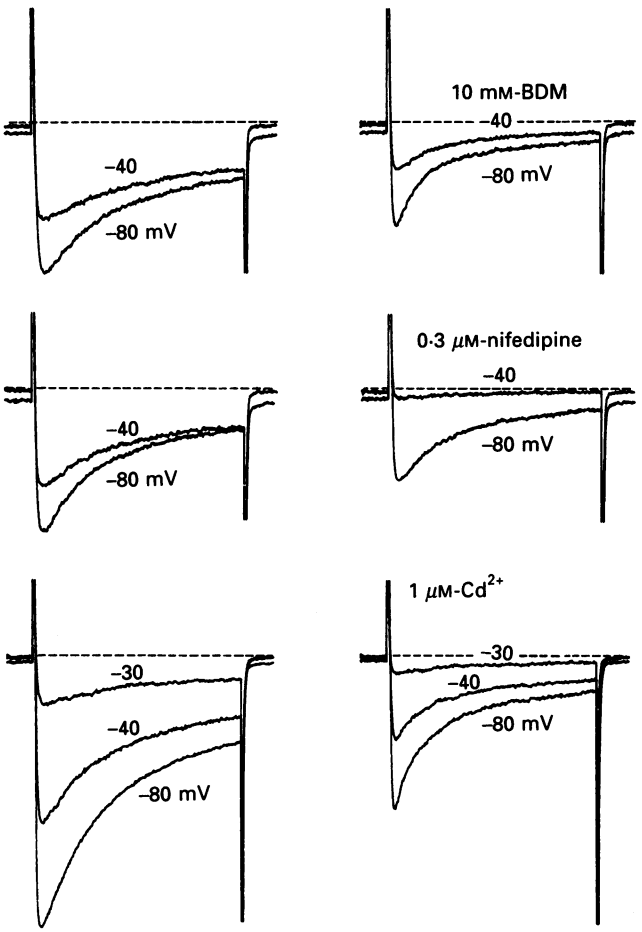


Fig. 9. The effect of holding potential on $I_{Ba}$ and the blocking actions of $Cd^{2+}$, nifedipine and BDM. Left panel of each row illustrates the voltage-dependence of $I_{Ba}$. The peak amplitude of $I_{Ba}$ at 0 mV, evoked from a holding potential of −40 mV, was approximately 60% of that recorded from −80 mV. Right panel of each row illustrates the voltage dependence of the $Ca^{2+}$ channel blockers. $I_{Ba}$ elicited from positive potentials (−40 or −30 mV) was more sensitive to the blocking actions of $Cd^{2+}$, BDM and particularly nifedipine.

From these shifts in the steady-state inactivation curves it was possible to estimate the dissociation constant for nifedipine and BDM binding to the resting and inactivated $Ca^{2+}$ channel, using the approach described by Bean *et al.* (1986). Assuming a one-to-one binding of these drugs to the resting and inactivated channel states, the dissociation constant for binding to the inactivated state ($K_I$) was calculated from

$$\Delta V_{0.5} = kL\{(1+[N]/K_I)/(1+[N]/K_R)\}$$

where $\Delta V_{0.5}$ is the shift of the mid-point of the steady-state inactivation curve, $k$ is

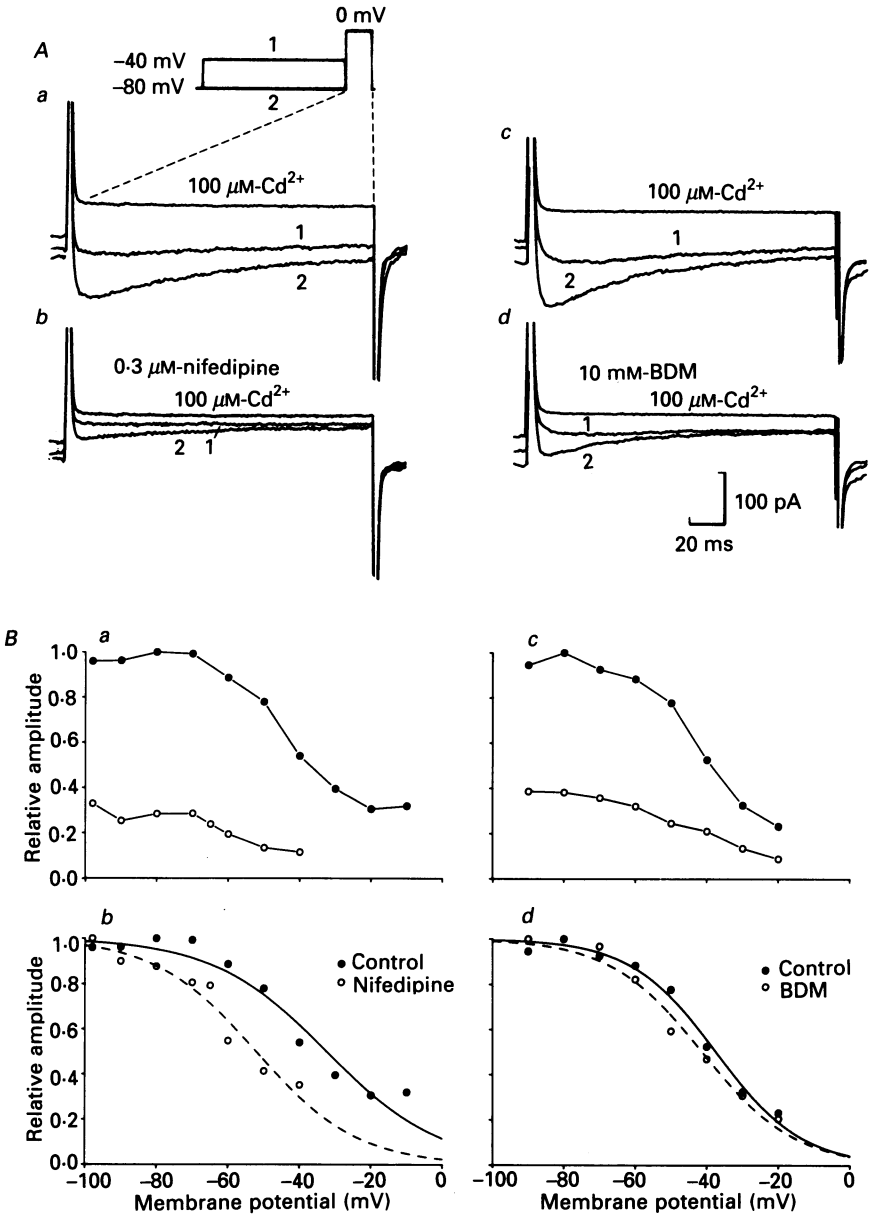


Fig. 10. Effects of 0·3 μM-nifedipine and 10 mM-BDM on the voltage-dependent inactivation of $I_{Ba}$ (7·5 mM-$Ba^{2+}$) using a twin-pulse protocol where conditioning pulses of various amplitudes and 1 s duration were followed by the test step (200 ms) to 0 mV; holding potential −80 mV. Stimulations were given in pairs with (trace 1) and without (trace 2) the conditioning pulse to correct for $Ca^{2+}$ channel current run-down. Protocols were then repeated in the presence of 100 μM-$Cd^{2+}$ to estimate the background membrane conductance. *A*, test currents to 0 mV in control (*Aa*, *c*), in 0·3 μM nifedipine (*Ab*) or 10 mM-BDM (*Ad*) without (2) and with a conditioning pulse to −40 mV (1). *B*, test $I_{Ba}$ in control after a conditioning pulse was expressed as a fraction of its accompanying conditioning-free $I_{Ba}$, then normalized as a fraction of their maximal value and plotted against potential. Test $I_{Ba}$ in the presence of $Ca^{2+}$ entry blockers was either expressed as a fraction of the averaged conditioning-free $I_{Ba}$ in control saline (*Ba*, *c*), or normalized (*Bb*, *d*).

the slope factor obtained experimentally, [N] is the concentration of the blocker used and $K_R$ is the dissociation constant of the blocker for the resting $Ca^{2+}$ channel at $-80$ mV. With $K_R = 1$ μM and $\Delta V_{0.5} = 19.7$ mV for nifedipine, $K_I$ was calculated as 78 nM; with BDM, $K_R = 10$ mM, $\Delta V_{0.5} = 4.7$ mV and $K_I = 5.1$ mM.

*Effects of nifedipine and BDM on $K^+$ contractures of intact strips of taenia caeci*

Voltage-dependent blockade of $Ca^{2+}$ channels by nifedipine and BDM could also be demonstrated in the intact strips of taenia caeci. $K^+$ contractures were elicited either by exposing muscle strips, bathed in a $Ca^{2+}$-free saline, to a 40 mM-$K^+$, 1·5 mM-$Ca^{2+}$ saline ($K^+$ concentration replacing an equimolar concentration of $Na^+$) (top panels in Fig. 11*A* and *B*) or by adding 1·5 mM-$Ca^{2+}$ to strips depolarized previously with a 40 mM-$K^+$, nominally $Ca^{2+}$-free saline (lower panels in Fig. 11*A* and *B*). If nifedipine (0·3 μM) was added just prior to the 40 mM-$K^+$, 1·5 mM-$Ca^{2+}$ solution the resulting contracture was smaller than the control contraction and phasic in nature. If, however, nifedipine was added to the depolarizing 40 mM-$K^+$, $Ca^{2+}$-free saline the contracture upon the addition of $Ca^{2+}$ was even more reduced (lower right panel, Fig. 11*A*). The addition of 10 mM-BDM to the depolarizing 40 mM-$K^+$, $Ca^{2+}$-free saline also produced a greater block of the contraction induced on the addition of 1·5 mM-$Ca^{2+}$ than that induced by 40 mM-$K^+$, 1·5 mM-$Ca^{2+}$ saline (Fig. 11*B*). These actions of BDM and nifedipine were fully reversible, as $K^+$ contractions returned on their removal (data not shown).

Inhibition of these $K^+$ contractures was expressed as a percentage of the area (first 2 min) of the control contraction in each strip. Nifedipine (0·3 μM) and BDM (10 mM) inhibited $46.5 \pm 5.5$ % ($n = 3$) and $70.7 \pm 3.9$ % ($n = 6$) respectively the contractions induced by the simultaneous addition of $K^+$ and $Ca^{2+}$; but inhibited $57.7 \pm 2.6$ % ($n = 3$) and $81.3 \pm 4.3$ % ($n = 6$) respectively the contractions induced by the addition of $Ca^{2+}$ to previously depolarized muscle strips.

*Bay K 8644 antagonizes BDM*

In embryonic chick heart cells the inhibitory action of BDM on the slow $Ca^{2+}$-dependent action potential can be antagonized by Bay K 8644, the $Ca^{2+}$ channel agonist (Sada, Sada & Sperelakis, 1985). We have therefore examined the effects of Bay K 8644 on $I_{Ba}$ in single cells of the taenia caeci. Bay K 8644 (1·0 μM) increased the amplitude of $I_{Ba}$ at 0 mV approximately 2- to 4-fold, this increase was reversed by 10 mM-BDM (Fig. 12*Aa*). Alternatively, the inhibitory action of 10 mM-BDM could be partially reversed by 0·1 and 1·0 μM-Bay K 8644 (Fig. 12*Ab*).

Bay K 8644 increased $I_{Ba}$ at all potentials. When plotted as a normalized conductance curve, $V_{0.5}$ of activation, for the cell in Fig. 12*Aa*, was $-16.7$ mV in the control saline and $-27.6$ mV in 1 μM-Bay K 8644 (data not shown). This agonist action of Bay K 8644 was also associated with a shift in the positive direction of the steady-state inactivation curve as estimated with the twin-pulse protocols described

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Application of nifedipine (0·3 μM) or BDM (10 mM) reduced the relative amplitude of $I_{Ba}$ at all conditioning potentials. Normalized data were fitted with Boltzmann distributions by least squares; $V_{0.5}$ was shifted from $-32.4$ to $-52.1$ mV in nifedipine (*Bb*), and from $-37.3$ to $-41.1$ mV in BDM (*Bd*).

above. In a separate cell, $I_{Ba}$ in 1 $\mu$M-Bay K 8644 was approximately four times larger than the averaged test $I_{Ba}$ (without a conditioning depolarization) in control saline (Fig. 12*B* and *C*). The addition of BDM (10 mM) antagonized these effects of Bay K 8644 (Fig. 12*B*). When normalized, the potential of half-maximal availability

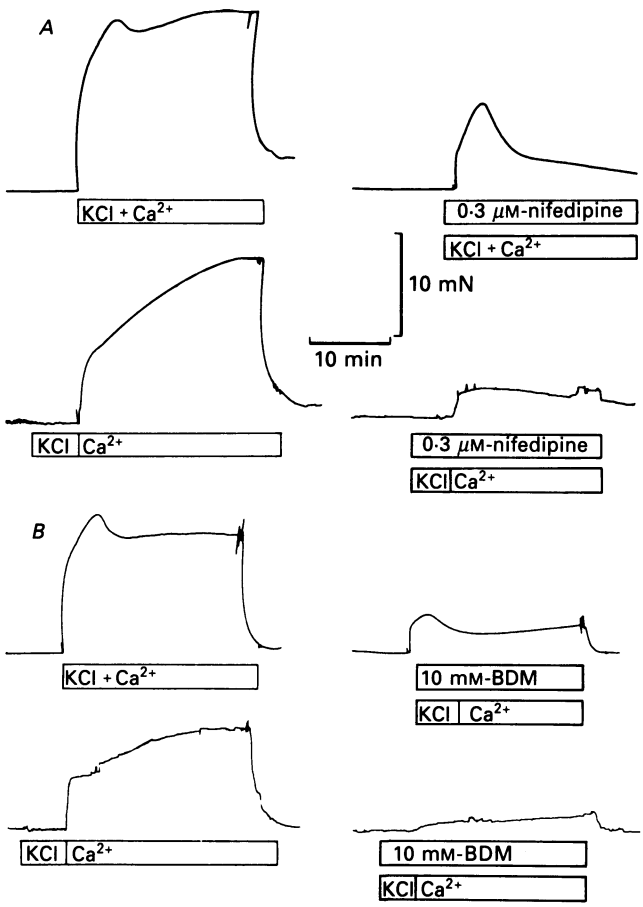


Fig. 11. Voltage-dependent inhibition of $K^+$ contractures in intact muscle strips by nifedipine (0·3 $\mu$M) and BDM (10 mM). *A*, $K^+$ contractures were elicited either by exposing muscle strips to a 40 mM-$K^+$, 1·5 mM-$Ca^{2+}$ saline (left column, top panel) or by adding 1·5 mM-$Ca^{2+}$ to strips depolarized previously (for 2 min) with 40 mM-$K^+$ in nominally $Ca^{2+}$-free saline (left column, lower panel). When nifedipine (0·3 $\mu$M) was added to the depolarizing 40 mM-$K^+$, $Ca^{2+}$-free solution the $K^+$ contracture upon the readmission of $Ca^{2+}$ was smaller than when nifedipine was added just prior to the 40 mM-$K^+$, 1·5 mM-$Ca^{2+}$ solution. *B*, voltage-dependent inhibition of $K^+$ contractions by BDM (10 mM); 10 mM-BDM also produced a greater inhibition of the $K^+$ contractions induced by the addition of 1·5 mM-$Ca^{2+}$ to the strips depolarized previously with 40 mM-$K^+$.

($V_{0·5}$) for control was $-38·4$ mV, $-26·4$ mV when in 1·0 $\mu$M Bay K 8644 and $-32·9$ mV when in Bay K 8644 + 10 mM-BDM (Fig. 12*C*).

*ATPγS and the action of BDM*

BDM was originally thought to have phosphatase-like activity (Wilson & Ginsberg, 1955; Green & Saville, 1956) and such an action has been evoked recently to explain

some of its inhibitory action in skeletal and cardiac muscle (Wiggins *et al.* 1980; Bergey *et al.* 1981; Fryer *et al.* 1988*b*). It may be that BDM is dephosphorylating $Ca^{2+}$ channels in smooth muscle which modifies their kinetics and/or number to produce the smaller, more rapidly inactivating $Ca^{2+}$ channel currents recorded. We

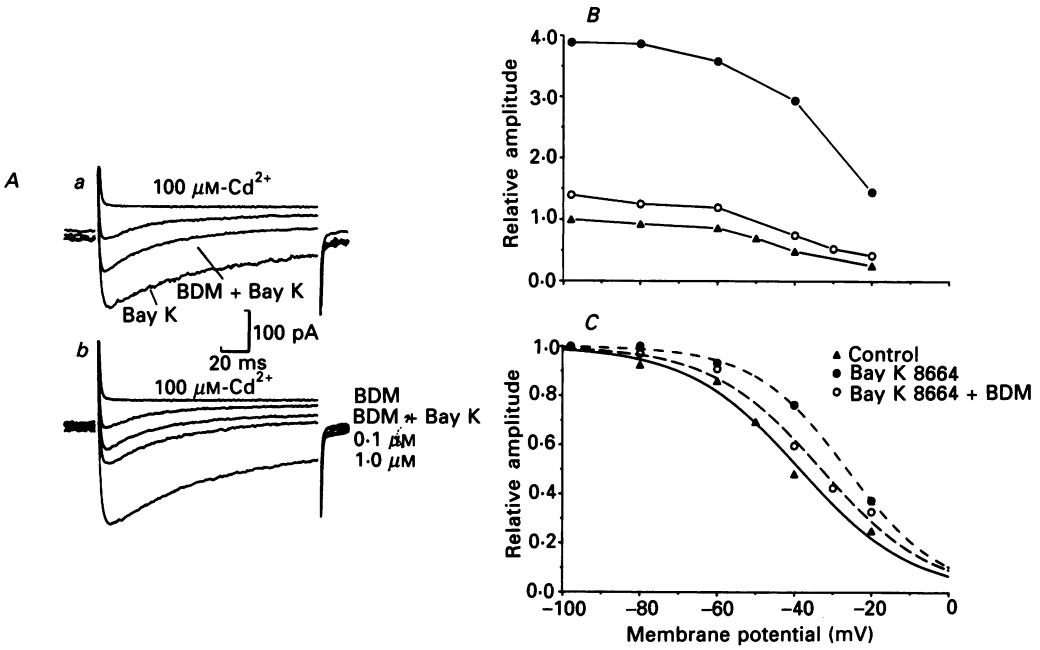


Fig. 12. Bay K 8644 antagonizes the inhibitory action of BDM. Bay K 8644 (1 μM) increased $I_{Ba}$ at 0 mV 2- to 4-fold. This increase was reversed upon the addition of 10 mM-BDM (*Aa*). The reduction of $I_{Ba}$ induced by 10 mM-BDM could also be reversed partially by 0·1 and 1·0 μM Bay K 8644 (*Ab*). Steady-state inactivation relationships, either expressed relative to the averaged test $I_{Ba}$ in control saline (*B*) or normalized to their own maximal fraction (*C*) have been plotted against potential. Bay K 8644 (1 μM) increased the relative amplitude of the test current at all potentials (*B*), which was associated with a shift in the positive direction of the normalized steady-state inactivation curve, fitted by least squares (*C*). BDM (10 mM) reversed partially the effects of Bay K 8644. $V_{0·5}$ in control, Bay K 8644 and Bay K 8644+BDM was −38·4, −26·4 and −32·9 respectively (*C*).

have examined this possibility by introducing into cells 1 mM-ATPγS, which should, in time, irreversibly thiophosphorylate cell proteins and therefore be resistant to the action of intrinsic phosphatases and BDM, if it is acting in a similar manner (Lang & Paul, 1989).

Cells were routinely perfused with 3 mM-ATP. The control for this set of experiments therefore was to add an additional 1 mM-ATP to the pipette solution. In Fig. 13*A* $I_{(peak)}$ and $I_{(400)}$ of $I_{Ba}$ in a cell perfused with 4 mM-ATP are plotted against time. Eight depolarizations to 0 mV (400 ms duration, 30 s apart) are separated by 3 min rest periods. In this cell there was substantial use dependence as evident by the progressive decrease in $I_{Ba}$ during the periods of stimulation; $Ca^{2+}$ channel run-down was also present but can be quantified by comparing the amplitude of $I_{Ba}$ at the beginning of each stimulation regimen in the absence of BDM (Fig. 13*A*).

When 1 mM-ATPγS was added to the internal solution of three cells the inhibitory action of 10 mM-BDM could not be prevented (Fig. 13*B*). ATPγS, however, had been able to enter cells as massive contractions were generally recorded within 5 min of rupture of the patch membrane, presumably due to the irreversible phosphorylation of the contractile proteins (Paul & Lang, 1989).

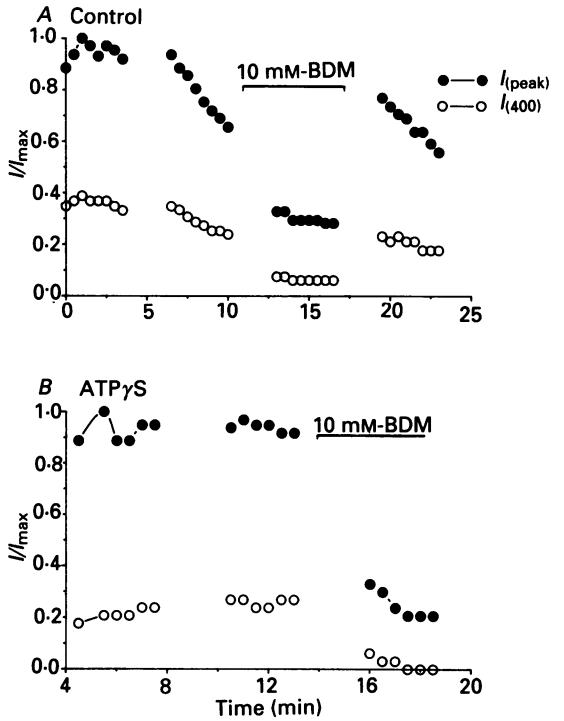


Fig. 13. The effects of the intracellular addition of ATPγS on the inhibition of $I_{Ba}$ by BDM. *A*, control cell (4 mM-ATP in pipette solution): 10 mM-BDM reduced both $I_{(peak)}$ and $I_{(400)}$ plotted against time. *B*, 1 mM-ATPγS, 3 mM-ATP added to the pipette solution did not prevent BDM (10 mM) reducing $I_{(peak)}$ and $I_{(400)}$.

## DISCUSSION

Tonic contractions of the guinea-pig taenia caeci and rat anococcygeus muscle, dependent on the influx of external $Ca^{2+}$ are blocked preferentially by BDM (5–30 mM) compared to the phasic component of the contraction (Fig. 9*A*; Wendt & Lang, 1987). BDM inhibition is also dependent on the prevailing membrane potential of the tissue as previous depolarization (with 40 mM-$K^+$) enhanced its action. The present experiments have therefore examined the mechanism of action of BDM on the $Ca^{2+}$ channel currents in single smooth muscle cells, comparing its action with the dihydropyridine, nifedipine and with another $Ca^{2+}$ entry blocker, $Cd^{2+}$.

All three agents reduced the $Ca^{2+}$ currents recorded in $Cs^+$-filled cells bathed in $Ca^{2+}$- or $Ba^{2+}$-containing solutions (7·5 mM), BDM and nifedipine being more effective when $Ca^{2+}$ acted as the charge carrier. The inhibitory action of nifedipine and, to a lesser extent, BDM was dependent on voltage, both being more effective at less negative holding potentials (Fig. 7). This voltage sensitivity arose from a shift

of 20 and 5 mV respectively in the negative direction of the steady-state inactivation curve in the presence of 0·3 $\mu$M-nifedipine and 10 mM-BDM (Fig. 8*Bb* and *d*). At higher concentrations, nifedipine (> 1 $\mu$M) also increased $Ca^{2+}$ channel currents at potential near threshold. This agonistic action could be quantified as a negative shift of 10 mV of the steady-state activation curve in 3 $\mu$M-nifedipine (Fig. 5*C*).

The modulated receptor hypothesis, which postulates that the state of the channel (rested, open or inactivated) is the main determinant of agonist–antagonist binding (Hille, 1977), has been used to investigate the action of a number of $Ca^{2+}$ channel blockers in a smooth muscle, particularly since the development of recording from single cells. Channel blockade mechanisms have been described as initial, conditioned (frequency or use dependent) or tonic. Initial block is defined as the block of rested channels which can be reversed if the tissue is subsequently hyperpolarized to negative potentials; e.g. (+)-isradipine action on $Ca^{2+}$ channels in myometrial smooth muscle (Honoré *et al.* 1989). Tonic block cannot be reversed by hyperpolarization; e.g. nifedipine action in the rabbit ear artery (Hering *et al.* 1988), spironolactone action in the rat portal vein (Dacquet, Loirand, Mironneau & Mironneau, 1987) and nicardipine action on the rabbit small intestine (Terada, Kitamura & Kuriyama, 1987). Conditioned block, on the other hand, is defined as the block that develops upon repetitive stimulation after the first stimulus, which can be relieved by a subsequent rest period at negative potentials and is though to reflect the binding of antagonists to open, or inactivated, channels which then removes them from the conducting channel pool; e.g. the action of verapamil and diltiazem on the rabbit small intestine (Terada *et al.* 1987), D600 on the rabbit ear artery (Hering, Bolton, Beech & Lim, 1989) and the main action of tiapamil on the guinea-pig bladder (Klöckner & Isenberg, 1986). Some agents show a mixed action; niguldipine produces an $\approx$ 60% initial block, followed by a further 20% conditioned block (Klöckner & Isenberg, 1989). In the present experiments all three blockers, after 2–3 min equilibrium with resting $Ca^{2+}$ channels, produced a block, which did not increase upon repetitive stimulation. Block was also not relieved by further 2–3 min rest periods so that the block induced by these three agents can be said to be tonic.

The acceleration of the decay of $I_{Ba}$ in the presence of BDM and $Cd^{2+}$, but not dramatically in nifedipine, was confirmed by scaling the amplitude of $I_{Ba}$ in the presence of the three blockers (at half-maximal concentrations) until they were superimposable with their control $I_{Ba}$ (data not shown). Recently, also, it has been shown that rapidly applying nifedipine during the decay phase of a prolonged $Ca^{2+}$ channel current, recorded in single ear artery cells of the rabbit, did not affect dramatically the subsequent current decay (Hering *et al.* 1988). This result is consistent with the lack of a conditioned block in the present experiments. Therefore, nifedipine appears not to bind rapidly to *open* $Ca^{2+}$ channels, but binds with a high affinity to rested channels as evident by the tonic block.

The action of nifedipine, however, does have a strong voltage dependence in the present experiments (Fig. 5*Bb*) and in the rabbit ear artery (Aaronson *et al.* 1988), moving the steady-state activation and inactivation curves in the negative direction. Such a voltage sensitivity has been interpreted previously as reflecting a high affinity of the blocker to the *inactivated* state of the $Ca^{2+}$ channel and a $K_I$ for nifedipine binding to the inactivated state was estimated as 78 nM (Bean *et al.* 1986). One might

have expected, however, that preferential binding to the inactivated state would have been revealed with a conditioned block of the $Ca^{2+}$ channels. In the present experiments, however, stimulus frequencies during repetitive stimulation were 0·03–0·05 Hz (holding potential −80 mV) to avoid an acceleration of $Ca^{2+}$ channel current run-down; perhaps slow enough to allow recovery from inactivation, even in the presence of nifedipine.

In cardiac tissue BDM (> 5 mM) reduced the plateau phase of the action potential and dephosphorylated cell membranes (Wiggins *et al.* 1980; Bergey *et al.* 1981), suggesting that BDM action was via a dephosphorylation of the slow $Ca^{2+}$ channels. It follows that if $Ca^{2+}$ channels in the smooth muscle cells of the taenia caeci are modulated by mechanisms involving phosphorylation/dephosphorylation BDM may be able to mimic the dephosphorylating branch of such an action. In the present experiments, BDM reduced $Ca^{2+}$ channel currents and $K^+$ contractures which, by analogy with cardiac cells (Hescheler, Mieskes, Rüegg, Takai & Trautwein, 1988) might be expected if $Ca^{2+}$ channels were being dephosphorylated (Kameyama, Hescheler & Trautwein, 1986). The action of BDM, however, could not be prevented by the thiophosphorylation of cell proteins with ATP$\gamma$S (1 mM) which would slow protein dephosphorylation (Fig. 11). Its action was also voltage dependent, which would not necessarily be expected if only a dephosphorylating mechanism was being evoked. In fact, the action of BDM was similar to nifedipine, could be antagonized by Bay K 8644 and perhaps suggests that BDM is acting at the dihydropyridine binding site on the $Ca^{2+}$ channel, but with a far lesser affinity.

We are grateful to Professor M. E. Holman for her valuable criticism of this manuscript. R.J.L. was supported by the NHMRC (Australia) and the Australian Kidney Foundation; R.J.P. was supported for part of this study by NIH HL23240, HL22619, and a Fogarty Senior International Fellowship.

## REFERENCES

AARONSON, P. I., BOLTON, T. B., LANG, R. J. & MACKENZIE, I. (1988). Calcium currents in single isolated smooth muscle cells from the rabbit ear artery in normal-calcium and high-barium solutions. *Journal of Physiology* **405**, 57–75.

BEAN, B. P. (1985). Two kinds of calcium channels in canine atrial cells. *Journal of General Physiology* **86**, 1–30.

BEAN, B. P., STUREK, M., PUGA, A. & HERMSMEYER, K. (1986). Calcium channels in muscle cells isolated from rat mesenteric arteries: modulation by dihydropyridine drugs. *Circulation Research* **59**, 229–235.

BENHAM, C. D., HESS, P. & TSIEN, R. W. (1987). Two types of calcium channels in single smooth muscle cells from rabbit ear artery studied with whole-cell and single-channel recordings. *Circulation Research* **61**, suppl. 1, 10–16.

BERGEY, J. L., REISER, J., WIGGINS, J. R. & FREEMAN, A. R. (1981). Oximes: enzymatic slow channel antagonists in canine cardiac Purkinje fibres? *European Journal of Pharmacology* **71**, 307–319.

BLANCHARD, E. M., ALPERT, N. R., ALLEN, D. G. & SMITH, G. L. (1988). The effects of 2,3-butanedione monoxime on the initial heat-tension–time-integral relation and aequorin light output from ferret papillary muscle. *Biophysical Journal* **53**, 605a.

DACQUET, C., LOIRAND, G., MIRONNEAU, C. & MIRONNEAU, J. (1987). Spironolactone inhibition of contraction and calcium channels in rat portal vein. *British Journal of Pharmacology* **92**, 535–544.

FRYER, M. W., GAGE, P. W., NEERING, I. R., DULHUNTY, A. F. & LAMB, G. D. (1988*a*). Paralysis of skeletal muscle by butanedione monoxime, a chemical phosphatase. *Pflügers Archiv* **411**, 76–79.

FRYER, M. W., NEERING, I. R. & STEPHENSON, D. G. (1988*b*). Effects of 2,3-butanedione monoxime on the contractile activation properties of fast- and slow-twitch rat muscle fibres. *Journal of Physiology* **407**, 53–75.

GANITKEVICH, V. YA., SHUBA, M. F. & SMIRNOV, S. V. (1986). Potential-dependent calcium inward current in a single isolated smooth muscle cell of the guinea-pig taenia caeci. *Journal of Physiology* **380**, 1–16.

GANITKEVICH, V. YA., SHUBA, M. F. & SMIRNOV, S. V. (1987). Calcium-dependent inactivation of potential-dependent calcium inward current in an isolated guinea-pig smooth muscle cell. *Journal of Physiology* **392**, 431–449.

GANITKEVICH, V. YA., SHUBA, M. F. & SMIRNOV, S. V. (1988). Saturation of calcium channels in single isolated smooth muscle cells of guinea-pig taenia caeci. *Journal of Physiology* **399**, 419–436.

GREEN, A. L. & SAVILLE, B. (1956). The reaction of oxime with isopropyl methylphosphonofluoridate (sarin). *Journal of the Chemical Society* **756**, 3887–3892.

HERING, E., BEECH, D. J., BOLTON, T. B. & LIM, S. P. (1988). Action of nifedipine and Bay K8644 is dependent on calcium channel state in single smooth muscle cells from rabbit ear artery. *Pflügers Archiv* **411**, 590–592.

HERING, E., BOLTON, T. B., BEECH, D. J. & LIM, S. P. (1989). Mechanism of channel block by D600 in single smooth muscle cells from rabbit ear artery. *Circulation Research* **64**, 928–936.

HESCHELER, J., MIESKES, G., RÜEGG, J. C., TAKAI, A. & TRAUTWEIN, W. (1988). Effects of a protein phosphatase inhibitor, okadaic acid, on membrane currents of isolated guinea-pig cardiac myocytes. *Pflügers Archiv* **412**, 248–252.

HILLE, B. (1977). Local anaesthetics: Hydrophilic and hydrophobic pathways for the drug-receptor reaction. *Journal of General Physiology* **69**, 497–515.

HIRST, G. D. S., SILVERBERG, G. D. & VAN HELDEN, D. F. (1986). The action potential and underlying ionic currents in proximal rat middle cerebral arterioles. *Journal of Physiology* **371**, 289–304.

HODGKIN, A. L. & HUXLEY, A. F. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. *Journal of Physiology* **117**, 500–544.

HONORÉ, E., AMÉDÉE, T., MARTIN, C., DACQUET, C., MIRONNEAU, C. & MIRONNEAU, J. (1989). Calcium channel current and its sensitivity to (+)isradipine in cultured pregnant rat myometrial cells. *Pflügers Archiv* **414**, 477–483.

KAMEYAMA, M., HESCHELER, J. & TRAUTWEIN, W. (1986). The protein-specific phosphatase 1 antagonizes the $\beta$-adrenergic increase of the cardiac Ca current. *Pflügers Archiv* **407**, 461–463.

KATZKA, D. A. & MORAD, M. (1989). Properties of calcium currents in guinea-pig gastric myocytes. *Journal of Physiology* **413**, 175–197.

KLÖCKNER, U. & ISENBERG, G. (1985). Calcium currents of cesium loaded isolated smooth muscle cells (urinary bladder of the guinea pig). *Pflügers Archiv* **405**, 340–348.

KLÖCKNER, U. & ISENBERG, G. (1986). Tiapamil reduces the calcium inward current of isolated smooth muscle cells. Dependence on holding potential and pulse frequency. *European Journal of Pharmacology* **127**, 165–171.

KLÖCKNER, U. & ISENBERG, G. (1989). The dihydropyridine niguldipine modulates calcium and potassium currents in vascular smooth muscle cells. *British Journal of Pharmacology* **97**, 957–967.

LANG, R. J. (1989). Identification of the major membrane currents in freshly dispersed single smooth muscle cells of guinea-pig ureter. *Journal of Physiology* **412**, 375–395.

LANG, R. J. (1990). The whole-cell $Ca^{2+}$-channel current in single smooth muscle cells of the guinea-pig ureter. *Journal of Physiology* **423**, 453–473.

LANG, R. J. & PAUL, R. J. (1989). Effects of butanedione monoxime, nifedipine and cadmium on Ca channel currents and isometric force in guinea pig taenia coli. *Proceedings of the XXXI International Congress of Physiological Sciences, Helsinki*, **528**.

LANG, R. J. & WENDT, I. R. (1987). Effects of 2,3-butanedione monoxime on barium currents recorded in single smooth muscle cells of the guinea-pig ureter. *Proceedings of the Australian Physiological and Pharmacological Society* **18**, 34*P*.

LANGTON, P. D., BURKE, E. P. & SANDERS, K. M. (1989). Participation of Ca currents in colonic electrical activity. *American Journal of Physiology* **257**, C451–460.

LEE, K. S. & TSIEN, R. W. (1983). Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialysed heart cells. *Nature* **302**, 790–794.

LEE, K. S. & TSIEN, R. W. (1984). High selectivity of calcium channels in single dialysed heart cells of the guinea-pig. *Journal of Physiology* **354**, 253–272.

LI, T., SPERELAKIS, N., TENEICK, R. E. & SOLARO, R. J. (1985). Effects of diacetyl monoxime on cardiac excitation–contraction coupling. *Journal of Pharmacology and Experimental Therapeutics* **232**, 688–695.

LOIRAND, G., PACAUD, P., MIRONNEAU, C. & MIRONNEAU, J. (1986). Evidence for two distinct calcium channels in rat vascular smooth muscle cells in short-term primary culture. *Pflügers Archiv* **407**, 566–568.

MAYLIE, D. R. & HUI, C. S. (1988). Effects of BDM on antipyrylazo III calcium signals in frog cut twitch fibres. *Biophysical Journal* **53**, 646a.

NAKAZAWA, K., SAITO, H. & MATSUKI, N. (1988). Fast and slowly inactivating components of Ca-channel current and their sensitivities to nicardipine in isolated smooth muscle cells from rat vas deferens. *Pflügers Archiv* **411**, 289–295.

OKABE, K., TERADA, K., KITAMURA, K. & KURIYAMA, H. (1987). Selective and long-lasting inhibitory actions of the dihydropyridine derivative, CV-4093, on calcium currents in smooth muscle. *Journal of Pharmacology and Experimental Therapeutics* **243**, 703–710.

PAUL, R. J. & LANG, R. J. (1989). Effects of okadaic acid and ATPγS on Ca channel currents in single enzymatically dispersed cells of guinea pig taenia coli. *Proceedings of the XXXI International Congress of Physiological Sciences, Helsinki*, **528**.

PAUL, R. J., WENDT, I. R. & LANG, R. J. (1989). Effects of 2,3-butanedione monoxime in single smooth muscle cells of the guinea-pig ureter and taenia coli. *FASEB Journal* **3**, A253.

SADA, H., SADA, S. & SPERELAKIS, N. (1985). The calcium channel agonist, Bay K-8644, antagonizes effects of diacetyl monoxime on cardiac tissues. *Canadian Journal of Physiology and Pharmacology* **63**, 1267–1270.

TERADA, K., KITAMURA, K. & KURIYAMA, H. (1987). Blocking actions of $Ca^{2+}$ antagonists on the $Ca^{2+}$ channels in the smooth muscle cell membrane of rabbit small intestine. *Pflügers Archiv* **408**, 552–557.

TSIEN, R. W., HESS, P., MCCLESKEY, E. W. & ROSENBERG, R. L. (1987). Calcium channels: Mechanisms of selectivity, permeation and block. *Annual Review of Biophysics and Biophysical Chemistry* **16**, 265–290.

WENDT, I. R. & LANG, R. J. (1987). Effects of 2,3-butanedione monoxime on smooth muscle contraction. *Proceedings of the Australian Physiological and Pharmacological Society* **18**, 33*P*.

WIGGINS, J. R., REISER, J., FITZPATRICK, D. F. & BERGEY, J. L. (1980). Ionotropic actions of diacetyl monoxime in cat ventricular muscle. *Journal of Pharmacology and Experimental Therapeutics* **212**, 217–224.

WILSON, I. B. & GINSBERG, S. (1955). A powerful reactivator of alkyl-phosphate-inhibited acetylcholinesterase. *Biochemica et Biophysica Acta* **18**, 168–175.

YABU, H., YOSHINO, M., SOMEYA, T., USUKI, T., OBARA, K. & TOSTUKA, M. (1989). Multiple subtypes of voltage dependent Ca channel in smooth muscle cells from guinea pig *taenia coli*. *Proceedings of the XXXI International Congress of Physiological Sciences, Helsinki*, **528**.

YAMAMOTO, Y., HU, S. L. & KAO, C. Y. (1989*a*). Inward current in single smooth muscle cells of the guinea pig taenia coli. *Journal of General Physiology* **93**, 521–550.

YAMAMOTO, Y., HU, S. L. & KAO, C. Y. (1989*b*). Outward current in single smooth muscle cells of the guinea pig taenia coli. *Journal of General Physiology* **93**, 551–564.

YATANI, A., SEIDEL, C. L., ALLEN, J. & BROWN, A. M. (1987). Whole-cell and single channel calcium currents of isolated smooth muscle cells from saphenous vein. *Circulation Research* **60**, 523–533.

YOSHINO, M., SOMEYA, T., NISHIO, A. & YABU, H. (1988). Whole-cell and unitary Ca channel currents in mammalian intestinal smooth muscle cells: evidence for the existence of two types of Ca channels. *Pflügers Archiv* **411**, 229–231.

YOSHINO, M., SOMEYA, T., NISHIO, A., YAZAWA, K., USUKI, T. & YABU, H. (1989). Multiple types of voltage dependent Ca channels in mammalian intestinal smooth muscle cells. *Pflügers Archiv* **414**, 401–409.