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Forskolin's structural analogue 1,9-dideoxyforskolin has Ca^{2+} channel blocker-like action in rat cerebellar granule cells

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Abstract

Forskolin, routinely used as a specific activator of the cAMP pathway, is also a blocker of various ionic channels in a cAMP-independent way. We investigated, in rat cerebellar granule cells in culture, the effects of forskolin and its structural analogue 1,9-dideoxyforskolin on Ca^{2+} entry. Changes in cytosolic free Ca^{2+} concentration ($[Ca]_i$) were monitored using fura-2 microfluorimetry. The increase in $[Ca]_i$ observed in response to membrane depolarization by 30 mM KCl was reduced by 20% in the presence of 100 μM forskolin, and by 71% with the same concentration of 1,9-dideoxyforskolin. A dose-response curve for 1,9-dideoxyforskolin gave an estimated IC_{50} of 54 μM. Additional experiments using the patch-clamp technique showed that 100 μM 1,9-dideoxyforskolin inhibit voltage-activated Ca^{2+} currents by 63%, although forskolin had no significant effect in the same conditions. This blocking effect of 1,9-dideoxyforskolin is not specific of a given Ca^{2+} channel type.

Keywords: Forskolin; 1,9-Dideoxyforskolin; Ca^{2+} current; Ryanodine store; Action potential; Cerebellar granule cell

1. Introduction

The initially observed cardiotonic action of forskolin (Lindner et al., 1978) was shown to result from its ability to activate adenylate cyclase (Seamon et al., 1981). Since then, forskolin has been used extensively as a specific activator of the cAMP pathway to study the role of this second messenger system in physiological responses. However, forskolin has additional actions that have been classified as non-cAMP-mediated effects, since they could be reproduced by the forskolin structural analogue, 1,9-dideoxyforskolin, which does not activate adenylate cyclase (reviewed in Laurenza et al., 1989). Some of these actions result from the direct interaction of the diterpenes with membrane proteins. They include inhibition of neuronal K^+ currents (Akins and McCleskey, 1993; Hoshi et al., 1988) and desensitization of nicotinic acetylcholine receptor (Wagoner and Pallotta, 1988). Anionic channels are also targets: kinetics of $GABA_A$ currents is changed by forskolin (White et al., 1992) and volume-activated Cl^- channels are blocked by 1,9-dideoxyforskolin (Diaz et al., 1993). In a previous paper we have shown that, in cerebellar granule cells, forskolin blocks both transient (I_A) and sustained K^+ currents in a cAMP-independent way (Zerr and Feltz, 1994) with an IC_{50} of 19 μM, close to the concentrations required to activate adenylate cyclase in these cells (Gonzalez et al., 1992; Wroblewska et al., 1993; Wu and Wojcik, 1986).

Since a blockade of voltage-activated K^+ channels is likely to modify the waveform of the action potential, we attempted to characterize the effect of these diterpenes on cellular excitability. Blockade of K^+ channels should result in an alterated contribution of Ca^{2+} currents to the action potential. Our experiments confirm that forskolin and 1,9-dideoxyforskolin prolong the action potential. Forskolin would thus not only activate adenylate cyclase, but also change intracellular Ca^{2+}. We therefore looked for the indirect effect of the blockade of K^+ channels by these diterpenes on Ca^{2+} entry evaluated from changes in intracellular Ca^{2+}. The Ca^{2+} entry following depolarization by 30 mM KCl should be altered when diterpenes are co-applied, if their blocking action on K^+ channels further depolarizes the cell. It turned out that both 1,9-dide-

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oxyforskolin and – to a lesser extent – forskolin inhibit the Ca^{2+} rise. In additional electrophysiological experiments we showed that, in the case of 1,9-dideoxyforskolin, but not for forskolin, this is the result of a direct blockade action on voltage-activated Ca^{2+} channels. A similar blocker-like action, obtained with 1,9-dideoxyforskolin, but not with forskolin, has been proposed in non-neuronal excitable cells (Abe and Karaki, 1992).

2. Materials and methods

2.1. Electrophysiology

Cerebellar granule cell cultures were obtained as previously described (De Waard et al., 1991) and recordings obtained after 4–7 days in vitro. Action potentials and whole cell Ca^{2+} currents were measured at room temperature (20–25°C) with an EPC-7 patch-clamp amplifier (List Electronics, Darmstadt, Germany). Recording pipettes were made from haematocrit glass capillaries (outer diameter: 1.5 mm) using a three-stage horizontal puller (Mecanex, Switzerland). They had a resistance of 4–8 MΩ with the solutions detailed below. Signals were low-pass filtered (3 kHz) before digitization. Linear leaks and residual capacitive currents were subtracted using on-line P/5 protocol (responses to five hyperpolarizing steps, each one-fifth of the test pulse, added to the experimental responses, leaving only the evoked current). Voltage- and current-clamp protocols, direct data storage on the computer and analysis were performed with the 5.1 version of the pClamp software (Axon Instruments, Foster City, USA). For membrane potential recordings the bath solution A was (in mM): NaCl 132, KCl 5, $CaCl_2$ 1, $MgCl_2$ 2, Hepes 10, glucose 10, pH 7.4/NaOH, 300 mosmol/l, and pipettes were filled with solution B: KCl 125, NaCl 5, $CaCl_2$ 1, $MgCl_2$ 2, EGTA 5.5, Hepes 10, $ATPNa_2$ 2, pH 7.2/KOH, 280 mosmol/l. For Ca^{2+} current recordings, the bath contained (solution C): Tris 120, TCA 110, TEACl 10, $BaCl_2$ 10, $MgCl_2$ 1, Hepes 10, glucose 10, pH 7.4/Tris, 310 mosmol/l, and pipette solution D was: Tris 100, $MgCl_2$ 2, $CaCl_2$ 3, EGTA 30, Hepes 100, CsCl 10, $ATPNa_2$ 2, pH 7.2/Tris, 290 mosmol/l.

2.2. $[Ca]_i$ measurements

The Ca^{2+} concentration was measured with the fluorescent probe fura-2, using an imaging system comprising a CCD camera (Extended ISIS, Photonic Science, UK) and an image analysis software (Starwise/Fluo 210, Imstar, France). Pairs of images were acquired every 5 s at excitation wavelengths of 350 nm and 380 nm, using a 500–530 nm bandpass emission filter. The $[Ca]_i$ values were calculated using the equation of Grynkiewicz et al. (1985). Calibration was performed on cells patched with pipettes containing various Ca-EGTA buffers together with 0.1 μM fura-2 (Augustine and Neher, 1992).

Cells were loaded for 60–80 min at room temperature with 2 μM fura-2/AM (Molecular Probes, USA) in the presence of 0.5% (w/v) bovine serum albumin dissolved in bath solution A. They were washed three times with fresh solution A before and after loading. During recording the cells were continuously superfused with either solution A or the test solution.

2.3. Drugs

Solutions were applied using perfusion barrels of ca. 100 μm diameter positioned ca. 100 μm from the recorded cell. Working solutions were prepared immediately before use from stock solutions, either stored at −10°C: forskolin (0.1 M in dimethylsulfoxide, DMSO), 1,9-dideoxyforskolin (0.1 M in DMSO), ω-conotoxin GVIA (10^{-4} M), or at 0°C: nicardipine (10^{-2} M in DMSO), carbamylcholine (0.1 M). Caffeine was freshly prepared each day. All the drugs were purchased from Sigma, except ω-conotoxin, which was from Almone Labs (Israel). Irreversible blockade of N-type Ca^{2+} channel was obtained with 1 μM ω-conotoxin applied for 15 min in a solution like A, where Ca^{2+} was replaced by Mg^{2+} (Marqueze et al., 1988). Final concentration of DMSO never exceeded 0.1%. Control experiments with 0.25% DMSO showed no effect on the responses.

Results are given as mean ± S.E.M. (number of cells). Comparisons of means were performed using the Student's t test for paired or unpaired data, as appropriate. The significance level chosen was $P = 0.05$.

3. Results

3.1. Effects of forskolin and 1,9-dideoxyforskolin on cell excitability

The resting potential of granule cells, estimated immediately after entering the whole-cell configuration, was -58.6 ± 0.9 mV ($n = 44$). Tetrodotoxin-sensitive action potentials were evoked by depolarizing current steps (300–500 pA). The firing threshold – defined as the inflection point of the potential trace, from which depolarization accelerates – was determined by calculating the first and second derivatives of each trace. Its average value was -29.7 ± 1.6 mV ($n = 11$). The potential peaked at 6.0 ± 2.8 mV ($n = 11$). In all cells, whatever the amplitude and the duration of the current step, a single action potential was recorded and no repetitive firing could be evoked. Application of either 100 μM forskolin or 100 μM 1,9-dideoxyforskolin changed the waveform of the action potential (Fig. 1). The firing threshold was not changed, but the peak of the action potential was further depolarized significantly (paired t test) by 1.8 ± 0.5 mV ($n = 4$) with forskolin and by 2.2 ± 1.0 mV ($n = 6$) with 1,9-dideoxyforskolin. The average duration (width at half-ampli-

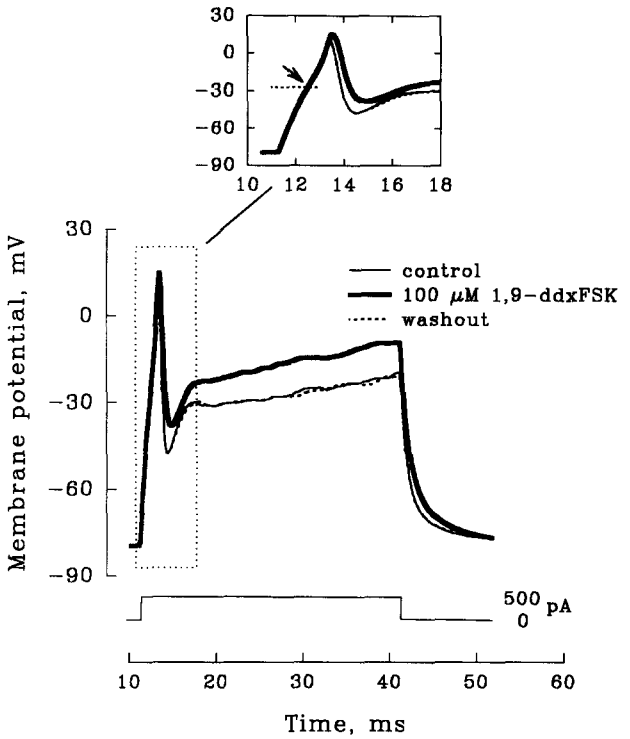

Fig. 1. Effect of 1,9-dideoxyforskolin (1,9-ddxFSK) on granule cells excitability. Superimposed traces recorded from soma membranes in control (thin curve), during (bold curve) and after removal (broken curve) of 100 μM 1,9-dideoxyforskolin. Action potentials were elicited by 500 pA current steps, 30 ms duration. The detailed action potential is shown in the inset. Note increased amplitude and duration of the spike in the presence of 1,9-dideoxyforskolin. The arrow and horizontal dotted line show the spike threshold.

tude from threshold to peak) of the action potential, 1.3 ± 0.1 ms ($n = 11$) in control, was prolonged significantly by 15 ± 5% ($n = 4$) with forskolin and by 35 ± 3% ($n = 6$) with 1,9-dideoxyforskolin. Neither diterpene modified significantly either the resting potential or the input resistance, which was measured by injecting constant hyperpolarizing current pulses ($n = 7$). On the other hand, the membrane chord resistance was increased at more depolarized potentials. For the cell illustrated in Fig. 1, at the end of the 30 ms depolarizing pulses of 500 pA, the membrane potential was −20 mV in control and −9 mV in the presence of 100 μM 1,9-dideoxyforskolin. This corresponds to an increase in the membrane chord resistance of 22 MΩ. On average, this increase was 15 ± 5% ($n = 4$) and 17 ± 3% ($n = 8$) with 100 μM forskolin and 1,9-dideoxyforskolin respectively. Again, no repetitive action potentials were ever elicited, despite the increased membrane resistance. On- and off-kinetics of the diterpene effect on cell excitability were similar. Maximal effect and recovery after washout were observed within 5 s. Blockade was sustained with longer applications of the diterpenes, and could be repeated several times on a given cell.

In a previous report we described that diterpenes inhibit voltage-activated K^+ currents in cerebellar granule cells (Zerr and Feltz, 1994). Our present results on the action potential are actually expected if voltage-activated K^+ currents are blocked. A further consequence of a longer spike may be a change in the Ca^{2+} entry occurring via the voltage-activated Ca^{2+} channels. In cerebellar granule cells, these channels are indeed activated by the action potential as shown by fluorometric estimates of internal Ca^{2+} concentration (Connor et al., 1987). More generally the Ca^{2+} entry evoked by a KCl-induced depolarization may be expected to be altered as an indirect consequence of K^+ channels blockade by diterpenes. Inhibition of the K^+ conductance should result in a larger depolarization, which may either facilitate or reduce Ca^{2+} entry, depending on the balance between activation and inactivation of Ca^{2+} channels.

3.2. Effects of forskolin and 1,9-dideoxyforskolin on Ca^{2+} entry

A first series of experiments was performed using the dye fura-2 to follow the variations of intracellular Ca^{2+} concentration ($[Ca]_i$) in response to cell depolarization by KCl. The mean resting $[Ca]_i$ was 31 ± 2 nM ($n = 67$). Superfusion with a saline solution containing 30 mM KCl (modified solution A with NaCl reduced by 30 mM to respect osmolarity) induced an average increase in $[Ca]_i$ of 148 ± 10 nM ($n = 67$; peak minus basal value). The rise in $[Ca]_i$ was smaller in the presence of diterpenes than with 30 mM KCl only (Fig. 2). This effect was reversible though recovery was only partial with the highest doses. The $[Ca]_i$ rise was reduced by 20 ± 2% ($n = 23$) in the presence of 100 μM forskolin and by 71 ± 3% ($n = 24$) with the same concentration of 1,9-dideoxyforskolin. Because this concentration was close to the limit of solubility for both drugs, a dose-response curve was only established for 1,9-dideoxyforskolin. The estimated half-maximal inhibition (IC_{50}) was 54 μM (Fig. 3).

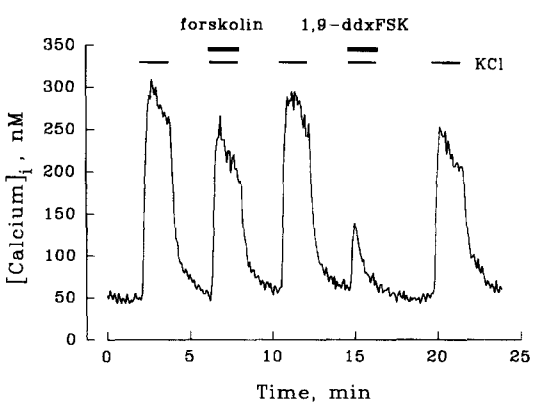

Fig. 2. Reduction by diterpenes of the $[Ca]_i$ increases following membrane depolarization by 30 mM KCl. Applications of KCl alone or simultaneously with 100 μM of either forskolin or 1,9-dideoxyforskolin (1,9-ddxFKS) are indicated by bars (100 s). The response to each co-application of drug and KCl is smaller than that to KCl alone immediately before. Note the larger blocking effect of 1,9-dideoxyforskolin.

Since an increase in $[Ca]_i$ induced by KCl can result from external Ca^{2+} entry through voltage-activated Ca^{2+} channels but also from liberation of Ca^{2+} from internal stores, via a Ca^{2+}-induced Ca^{2+} release mechanism, we performed some experiments to look for the effects of diterpenes on Ca^{2+} stores. Both ryanodine-sensitive (De Erausquin et al., 1992) and IP_3-sensitive (Fohrman et al., 1993) Ca^{2+} stores are present in cerebellar granule cells. In these cells, caffeine activates the release of Ca^{2+} from ryanodine stores only after depolarizing conditions promoting Ca^{2+} entry and storage (De Erausquin et al., 1992). Similarly, as shown by Fohrman et al. (1993), activation of the muscarinic m3 receptor in cerebellar granule cells specifically induces the release of Ca^{2+} from IP_3-activated Ca^{2+} stores, provided they were previously loaded by cell depolarization with KCl.

To examine the possibility of a direct action of 1,9-dideoxyforskolin on the internal stores, we therefore continuously superfused the cells with solutions containing 30 mM KCl, which resulted in a higher stabilized level of $[Ca]_i$ (81 ± 4 nM, $n = 29$) after the initial transient increase (Fig. 4A and Fig. 4B). Application of 10 mM caffeine for 25 s induced a transient increase in $[Ca]_i$ of 50 ± 6 nM ($n = 15$, Fig. 4A). When 100 μM 1,9-dideoxyforskolin was co-applied with caffeine, this $[Ca]_i$ change was not significantly modified (41 ± 7 nM, $n = 15$). Thus, in these conditions, the liberation of Ca^{2+} from ryanodine stores is not affected by 1,9-dideoxyforskolin. Similarly, following activation of muscarinic receptors with 20 s applications of 100 μM carbachol during prolonged superfusion with 30 mM KCl, the mean increase in $[Ca]_i$ was 147 ± 26 nM ($n = 14$) and did not significantly change when 100 μM 1,9-dideoxyforskolin was co-applied with carbachol (132 ± 25 nM, $n = 14$, Fig. 4B). However the absence of effect of 1,9-dideoxyforskolin on the response to caffeine or carbachol may be due to a diffusion delay for 1,9-dideoxyforskolin to reach the reticulum membrane. Therefore we applied the same stimuli after a continuous exposure to 1,9-dideoxyforskolin. In this case caffeine or carbachol was no longer able to elicit Ca^{2+} release, or induced a much reduced release when applied shortly after 1,9-dideoxyforskolin (Fig. 4B). However these experi-

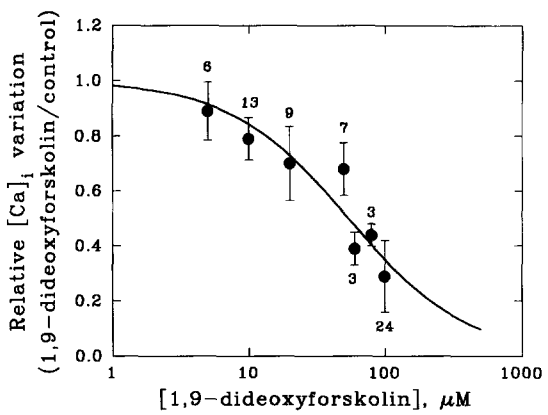

Fig. 3. Dose-response curve of the action of 1,9-dideoxyforskolin on 30 mM KCl-induced $[Ca]_i$ responses. In a series of KCl applications, the $[Ca]_i$ variations induced by co-application with 1,9-dideoxyforskolin were normalized to the previous $[Ca]_i$ increase (with KCl only). Symbols represent mean values and error bars show S.E.M. The number of cells studied in each case is indicated. The continuous line shows the curve fitted according to the equation:

$$\text{relative}\,[Ca]_i\,\text{variation} = IC_{50}/(IC_{50} + [1,9\text{-dideoxyforskolin}])$$

where IC_{50}, the concentration producing half-maximal inhibition, is equal to 54 μM.

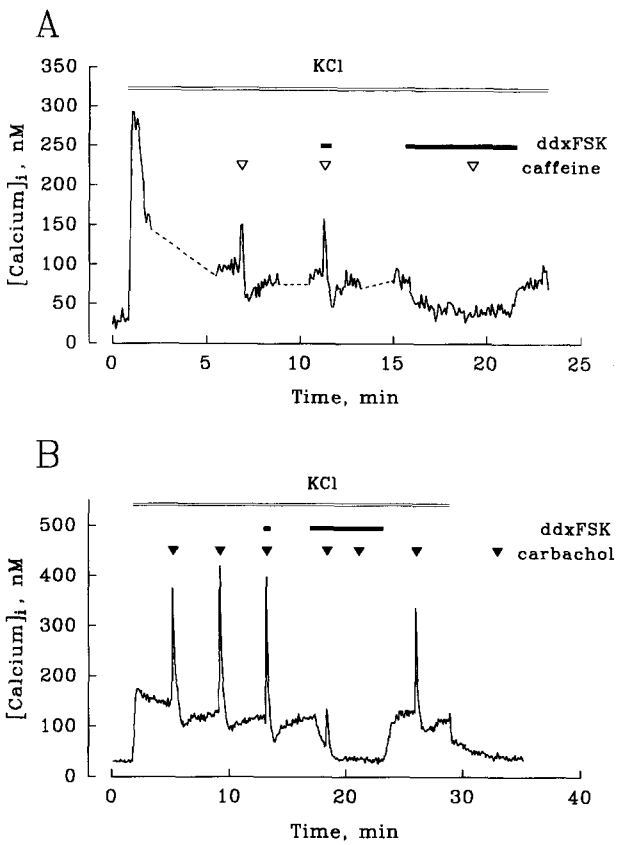

Fig. 4. Effect of 1,9-dideoxyforskolin (ddxFSK, black bar) on the release of Ca^{2+} from internal stores. To fill both ryanodine-sensitive (A) and IP_3-sensitive (B) Ca^{2+} stores, resting $[Ca]_i$ was raised by maintaining 30 mM KCl in bath solution (open bars). A: liberation of Ca^{2+} from ryanodine-sensitive stores was induced by successive 10 mM caffeine applications (▽, 25 s). Caffeine alone or co-applied with 100 μM 1,9-dideoxyforskolin yielded a similar rise in $[Ca]_i$. No response to a third application of caffeine was observed after prolonged exposure to 1,9-dideoxyforskolin that decreased basal $[Ca]_i$. Dashed lines correspond to interruptions in data acquisition. B: release of Ca^{2+} from IP_3-sensitive stores was induced by 20 s applications of 100 μM carbachol (▼). The $[Ca]_i$ increase induced by carbachol when 100 μM 1,9-dideoxyforskolin was simultaneously applied (third application) was not significantly different from that in control (first two applications). Again, when 1,9-dideoxyforskolin was applied continuously, $[Ca]_i$ was decreased and carbachol was no longer able to elicit Ca^{2+} release from IP_3-sensitive stores; the effect of carbachol was recovered after washing out 1,9-dideoxyforskolin, which results in a higher $[Ca]_i$ (sixth carbachol application). In the presence of 5 mM KCl in the bathing medium, the resting value of $[Ca]_i$ was also lower, and carbachol also failed to release Ca^{2+} (last application).

ments do not allow to conclude a direct effect of 1,9-dideoxyforskolin on the reticulum channels. Indeed, prolonged exposure to 1,9-dideoxyforskolin is accompanied by a decrease of $[Ca]_i$ back to a low level (39 ± 3 nM, $n = 29$), at which neither caffeine nor carbachol is able to elicit a change in $[Ca]_i$ (De Erausquin et al., 1992; Fohrman et al., 1993). In this respect the last application of carbachol in figure 4B illustrates that no response is observed in the presence of 5 mM KCl, at a low level of $[Ca]_i$.

3.3. Effects of forskolin and 1,9-dideoxyforskolin on Ba^{2+} currents

The possibility of a direct effect of diterpenes on Ca^{2+} channel activity was examined with the patch-clamp technique using Ba^{2+} as charge carrier. Ba^{2+} currents (I_{Ba}) have already been characterized in cultured cerebellar granule cells (De Waard et al., 1991; Marchetti et al., 1991). Typical current traces elicited by voltage jumps to −10 mV (Fig. 5A, inset) and current/voltage (I/V) relationship (Fig. 5B, open circles) showed kinetics and voltage dependency, respectively, similar to those already described.

Forskolin itself, at concentrations of up to 100 μM, had no detectable effect on I_{Ba} ($n = 7$; one cell illustrated in Fig. 5A, filled squares). As illustrated in Fig. 5A, 100 μM 1,9-dideoxyforskolin reversibly reduced the amplitude of I_{Ba} evoked at −10 mV from a resting potential of −80 mV. In these conditions, the mean current inhibition was 63 ± 3% ($n = 12$). To analyze the voltage dependency of this effect, I_{Ba} was measured at test potentials ranging from −40 to +50 mV. The resulting I/V relationship (Fig. 5B) shows that 1,9-dideoxyforskolin inhibited I_{Ba} at all the potentials tested. On average the maximal cellular conductance (G_{max}) was reduced from 2.2 ± 0.3 nS to 0.6 ± 0.1 nS ($n = 3$). The half-activation potential, V_{50}, was not modified (−21 ± 4 mV in control vs. −16 ± 4 mV with 1,9-dideoxyforskolin).

In cerebellar granule cells several types of high threshold Ca^{2+} currents have been identified on a pharmacological basis. N-type and L-type currents can be inhibited by ω-conotoxin and nicardipine respectively, and thus separated from other components (P- and Q-type). The effect of 100 μM 1,9-dideoxyforskolin on the current amplitude was tested by recording I_{Ba} successively in the absence (control) or in the presence of 1,9-dideoxyforskolin, either on non-treated cells (total current) or on cells pre-treated respectively with 1 μM ω-conotoxin (leaving L- and other types), 10 μM nicardipine (leaving N- and other types), or both channel blockers (recording only other types).

In non-treated cells the amplitude of I_{Ba} at −10 mV was −167 ± 15 pA ($n = 12$) in control, and was decreased to −63 ± 9 pA by 100 μM 1,9-dideoxyforskolin (Fig. 6). In the presence of ω-conotoxin I_{Ba} was −114 ± 20 pA ($n = 9$), and was brought down to −42 ± 10 pA by 1,9-dideoxyforskolin. Similarly, with nicardipine, I_{Ba} was −112 ± 17 pA in control and −40 ± 7 pA with 1,9-dideoxyforskolin ($n = 5$). Finally, with both channel blockers, the non-N-non-L (other type) I_{Ba} was reduced from −45 ± 5 to −24 ± 2 pA ($n = 4$) by the diterpene.

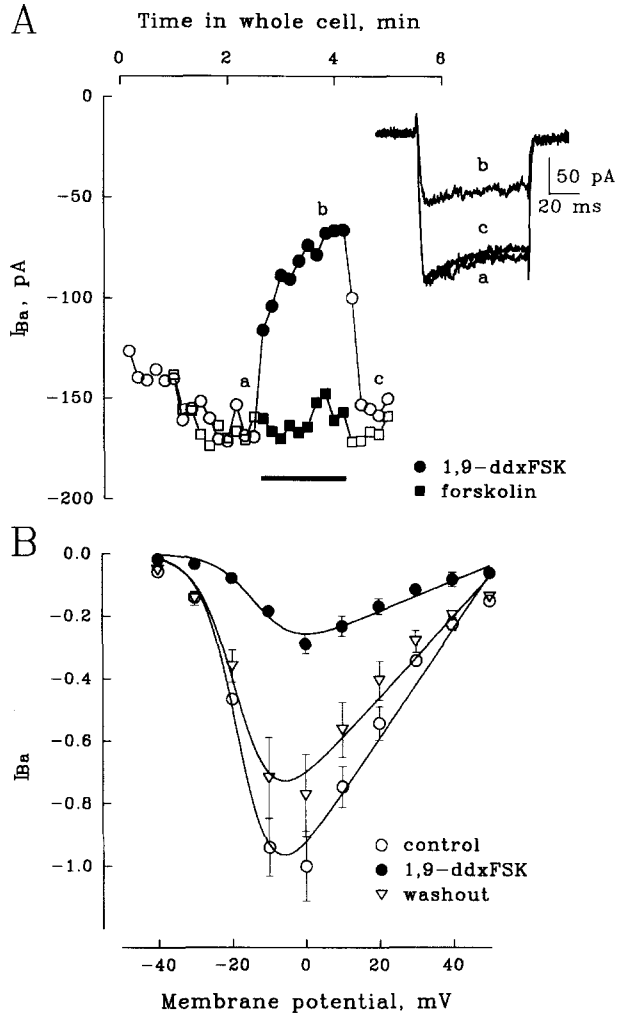

Fig. 5. Effects of forskolin and 1,9-dideoxyforskolin (1,9-ddxFSK) on I_{Ba}. A: I_{Ba} was evoked by depolarizing voltage steps to −10 mV from a holding potential of −80 mV. Current amplitudes were measured as the mean plateau current at the end of the 75 ms pulse and plotted against time in the whole cell recording mode. Measures were taken every 10 s. Horizontal bar corresponds to application of 100 μM of either forskolin (■) or 1,9-dideoxyforskolin (●) on two different cells. Inset: the illustrated superimposed I_{Ba} traces were obtained before, during and after 1,9-dideoxyforskolin application at times a, b and c as indicated on the graph. B: current-voltage relationships recorded in control (○), 100 μM 1,9-dideoxyforskolin (●) and washout (▽) conditions (average of three cells; bars: ±S.E.M.). Data were normalized with respect to the mean maximal current recorded in control (−131 ± 15 pA, $n = 3$, at 0 mV). Data points were fitted according to the Boltzmann equation:

$$I_{Ba} = G_{max} \frac{V - E_{Ba}}{1 + \exp\left(-\frac{V - V_{50}}{k}\right)}$$

where G_{max} is the maximal conductance, E_{Ba} the equilibrium potential for Ba, V_{50} the half-activation potential, and k the slope coefficient.

The contributions of N-type and L-type currents can be estimated after subtraction of the remaining other types

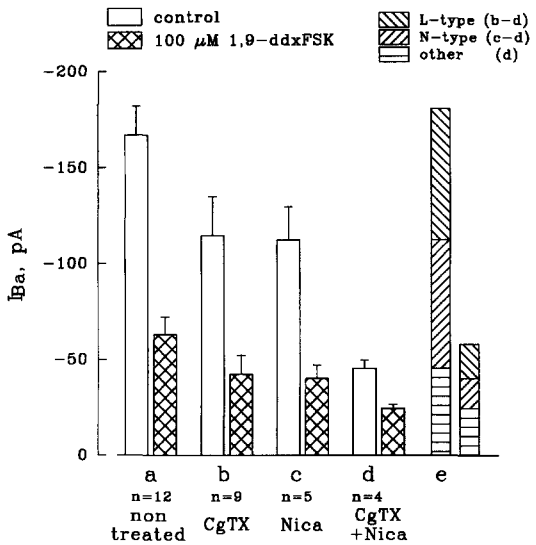

Fig. 6. Estimation of the blocking effect of 1,9-dideoxyforskolin (1,9-ddxFSK) on Ba currents. The mean current amplitudes (±S.E.M.) at −10 mV in the presence of 1,9-dideoxyforskolin (cross-hatched bars) are compared to the corresponding control conditions (open bars): non-treated cells (a); after blocking N-type component by 1 μM ω-conotoxin (CgTX, b); after blocking the L-type component by 10 μM nicardipine (Nica, c); in the presence of both channel blockers (d). All three components of I_{Ba} were clearly inhibited by 1,9-dideoxyforskolin (e). Total currents reconstructed from their components (e; using b, c and d) were not significantly different from experimentally observed currents (a).

from the currents recorded in the presence of nicardipine or ω-conotoxin respectively. N-type, L-type and other currents represent about 32, 33 and 35% of the total current in control, respectively. In all four conditions, the addition of 100 μM 1,9-dideoxyforskolin induced a significant inhibition of I_{Ba}. 1,9-Dideoxyforskolin has a blocking effect on all components and does not appear to inhibit specifically one type of Ca^{2+} current.

4. Discussion

Recent review (Laurenza et al., 1989) has underlined the multiple side-effects of forskolin that lead to a non-specific blockade of many ionic permeabilities. Our results provide evidence that, at concentrations up to 100 μM, forskolin leaves intact voltage-dependent Ca^{2+} entries, although we showed that it blocks K^+ currents in a previous paper (Zerr and Feltz, 1994). On the contrary, we show that the forskolin structural analogue 1,9-dideoxyforskolin is a potent blocker of Ca^{2+} currents.

4.1. Diversity of forskolin's actions on cerebellar granule cells

In the first part of this study, we were looking for the consequences on the cellular excitability of the direct blocking action of forskolin on K^+ channels (Zerr and Feltz, 1994). We show that forskolin increases the action potential duration without affecting resting potential values. These results are compatible with the blockade of voltage-activated K^+ channels. There are already indications that forskolin prolongs the action potential of neurons by reducing voltage-activated K^+ currents (Dunlap, 1985; Grega and Macdonald, 1987). However, in these cases, the authors ascribed this effect of forskolin to its activation of adenylate cyclase, since it could be reproduced by application of compounds that stimulate the cAMP pathway. Actually, PKA-dependent phosphorylation was shown to inhibit Ca^{2+}-activated K^+ channels (Pedarzani and Storm, 1993; Reinhart et al., 1991), and to reduce transient and sustained K^+ currents (Hochner and Kandel, 1992). Two distinct mechanisms – one independent (present work), one dependent on cAMP – can therefore account for spike prolongation after forskolin application.

In addition we show that forskolin reduces the rise in intracellular Ca^{2+} concentration induced by depolarization with KCl. Since no direct inhibition of Ca^{2+} currents was observed in voltage-clamp experiments, this effect on Ca^{2+} entry may result indirectly from the blockade of K^+ channels: a more depolarized potential may cause a faster inactivation of Ca^{2+} channels. Another explanation may be an additional effect of forskolin either in reducing Ca^{2+} sources (transmembrane currents or intracellular pools) or stimulating Ca^{2+} pumping. A modulation of the Ca^{2+} transport pathways may occur either directly, or indirectly via a PKA-dependent phosphorylation. Indeed forskolin is known to activate adenylate cyclase in cerebellar granule cells (Gonzalez et al., 1992; Wroblewska et al., 1993; Wu and Wojcik, 1986), and thus the reduced KCl-induced $[Ca]_i$ elevation could occur through a cAMP-mediated mechanism.

4.2. Blockade of Ca^{2+} currents by 1,9-dideoxyforskolin

1,9-Dideoxyforskolin is a structural analogue of forskolin currently used to study the non-cAMP mediated effects of forskolin. We report here that 1,9-dideoxyforskolin – but not forskolin – inhibits neuronal Ba^{2+} currents. This supports the fact that although forskolin and 1,9-dideoxyforskolin are structural analogues, their effects can diverge. In the same way, 1,9-dideoxyforskolin inhibits the contraction induced by K^+ more strongly than forskolin through a Ca^{2+} channel blocker-like action in vascular smooth muscles (Abe and Karaki, 1992). 1,9-Dideoxyforskolin but not forskolin markedly inhibited depolarization-evoked ^{45}Ca flux in PC12 cells (Nishizawa et al., 1990). On the other hand, an inhibitory effect of forskolin on cardiac cells Ca^{2+} current, that was not a cAMP-dependent phosphorylation mechanism, could not be reproduced by 1,9-dideoxyforskolin (Boutjdir et al., 1990).

The effect of 1,9-dideoxyforskolin on Ca^{2+} current that we describe adds to the already complex pharmacology of

Ca^{2+} channels (Hosey and Lazdunski, 1988). These are blocked by molecules that belong to different chemical families and our results extend this blocking effect to diterpenes. An interesting point is that in addition to its ability to block L- and N-type Ca^{2+} currents (this work), 1,9-dideoxyforskolin is also able to block the transient K^+ current (Zerr and Feltz, 1994). A similar direct inhibitory action of more classical Ca^{2+} channel blockers on plant K^+ channels has been described (Thomine et al., 1994). Such observations may not be specific to plant K^+ currents, since nicardipine, an L-type Ca^{2+} current blocker, inhibits an outward rectifying K^+ current in cerebellar granule cells (Fagni et al., 1994). These overlaps in the respective pharmacology of the K^+ and Ca^{2+} channels possibly point to structural analogies.

Our results, both from microfluorometry and from patch-clamp experiments, clearly show that 1,9-dideoxyforskolin has a blocking effect on voltage-activated Ca^{2+} channels of the plasma membrane. As to IP_3- and ryanodine-sensitive release channels associated with internal Ca^{2+} stores, our present experiments let the possibility of a similar inhibitory action of 1,9-dideoxyforskolin open. Short exposures to 1,9-dideoxyforskolin do not prevent Ca^{2+} release stimulated by caffeine or carbachol. However, this may be due to a diffusion delay in the effect of 1,9-dideoxyforskolin, since longer applications prevent this release. On another hand, the absence of response to caffeine or carbachol during prolonged exposures to 1,9-dideoxyforskolin may also be the result of the corresponding lower cytosolic Ca^{2+} level and/or a depletion of internal Ca^{2+} stores in these conditions. To address this question, further experiments are required that involve controlling $[Ca]_i$ without interfering with either voltage-dependent Ca^{2+} channels or the repletion state of internal stores.

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