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Inhibition of recombinant human cardiac L-type Ca^{2+} channel α_{1C} subunits by 3-isobutyl-1-methylxanthine

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Abstract

Inhibition of ion channels by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) and related compounds has been demonstrated in various cell types, including the neuromuscular junction, GH_3 cells and vascular smooth muscle cells. These effects may be unrelated to the actions of these compounds on cellular metabolism, intracellular Ca^{2+} stores and phosphodiesterase inhibition. In this study, the inhibition of recombinant human cardiac L-type Ca^{2+} channel α_{1C} subunits by IBMX was examined using the whole-cell configuration of the patch clamp technique. Inhibition was repeatable, voltage-independent and associated with increased apparent channel inactivation. The actions of IBMX were unaffected in the presence of inhibitors of protein kinases A and G. The non-xanthine phosphodiesterase inhibitor rolipram had a small inhibitory effect on currents, but this was also unaffected by a protein kinase A inhibitor. These effects of IBMX could not be attributed to release of Ca^{2+} from intracellular stores. Our findings indicate that methylxanthines can inhibit the cardiac L-type Ca^{2+} channel α_{1C} subunit in the absence of auxiliary subunits by an undetermined, possibly direct mechanism.

Keywords: Ca^{2+} channel; Isobutylmethylxanthine; Phosphodiesterase; Cyclic nucleotide

1. Introduction

The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) is one of a group of methylxanthine compounds widely used in cellular signal transduction studies. These compounds have numerous effects on cellular metabolism, including liberation of Ca^{2+} from intracellular stores (Verkhratsky and Shmigol, 1989; Usachev and Verkhratsky, 1995), antagonism of adenosine receptors (Daly et al., 1981; Dolphin et al., 1986; Choi et al., 1988) and, perhaps most commonly, elevation of cyclic nucleotide (cAMP and cGMP) levels through inhibition of phosphodiesterase (Butcher and Sutherland, 1962; Choi et al., 1988). More recently, evidence has emerged for IBMX and related compounds to interfere with ionic channels via mechanisms which do not involve the above-described actions, and may well be direct effects. Thus, for example, IBMX has been shown to prolong the time course of miniature end-plate currents at the neuromuscular junction (Silinsky and Vogel, 1987) and caffeine reduces depolarization-mediated ^{45}Ca influx into vascular smooth muscle cells via a mechanism unrelated to intracellular Ca^{2+} stores or cAMP levels (Leitjen and Van Breemen, 1984). More recently, both caffeine and IBMX have been shown to inhibit L-type Ca^{2+} channel currents in isolated vascular smooth muscle cells by a mechanism which does not depend on intracellular Ca^{2+} levels and is not mimicked by non-xanthine phosphodiesterase inhibitors (Hughes et al., 1990). These agents have also been shown to inhibit sustained Ca^{2+} currents in clonal pituitary (GH_3) cells (Simasko and Yan, 1993).

In the present study, we have investigated the interaction of IBMX with recombinant cardiac Ca^{2+} channels. We have developed a clonal human embryonic kidney (HEK 293) cell line stably expressing the human cardiac L-type Ca^{2+} channel α_{1C} subunit (Schultz et al., 1993, Fearon et al., 1997), which forms the ionic pore and possesses the voltage sensor (Hofmann et al., 1994). Step depolarizations applied to these cells evoke dihydro-

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pyridine-sensitive inward currents carried by Ba^{2+}, in the absence of auxiliary (β and $\alpha_2-\delta$) subunits (Fearon et al., 1997). Our results suggest that IBMX can inhibit these currents via a direct interaction with the channel protein.

2. Materials and methods

A 7025 base pair *Not*I/*Hin*dIII fragment encoding the ORF of the human L-type Ca^{2+} channel α_{1C} cDNA (11) was subcloned into pREP9 (Invitrogen) from pBluescript SK- using standard techniques (Sambrook et al., 1989). Correct identity of the new expression construct was confirmed by restriction analysis and DNA sequencing. HEK 293 cells were grown in minimum essential medium containing 9% fetal calf serum (Globepharm), 1% non-essential amino acids, gentamycin (100 mg/l), penicillin G (10^5 U/l) streptomycin (100 mg/l) and amphotericin B (0.25 mg/l). Cells were grown in 75 cm^2 flasks and were transfected with 15 μg pREP9hα_{1C} using 34 μl Tfx-50 lipid reagent (Promega) in fetal calf serum-deficient medium for 1 h. The following day, cells were rinsed with phosphate-buffered saline (PBS), dissociated with 0.05% trypsin and 0.5 mM EDTA in PBS and diluted into six 225 cm^2 flasks. Three days after transfection, selection for transformants was applied by supplementing the medium with 400 mg/l G418 (Gibco). Six weeks later, independent G418-resistant foci were isolated with cloning cylinders and subcultured separately. Expression of α_{1C} was found in 12 of 15 isolated clones using whole-cell patch clamp recordings (Hamill et al., 1981; see below for details). The present studies were conducted using a single clone (number 14).

The whole-cell patch clamp technique was used to record current flowing through α_{1C} subunits in transfected HEK 293 cells. Pipettes (4–8 MΩ resistance) were filled with (in mM): CsCl 120, tetraethylammonium-Cl 20, $MgCl_2$ 2, EGTA 10, HEPES 10, ATP 2 (pH 7.2), and cells were continually perfused at a rate of approximately 1–2 ml min^{-1} with a solution composed of (in mM):

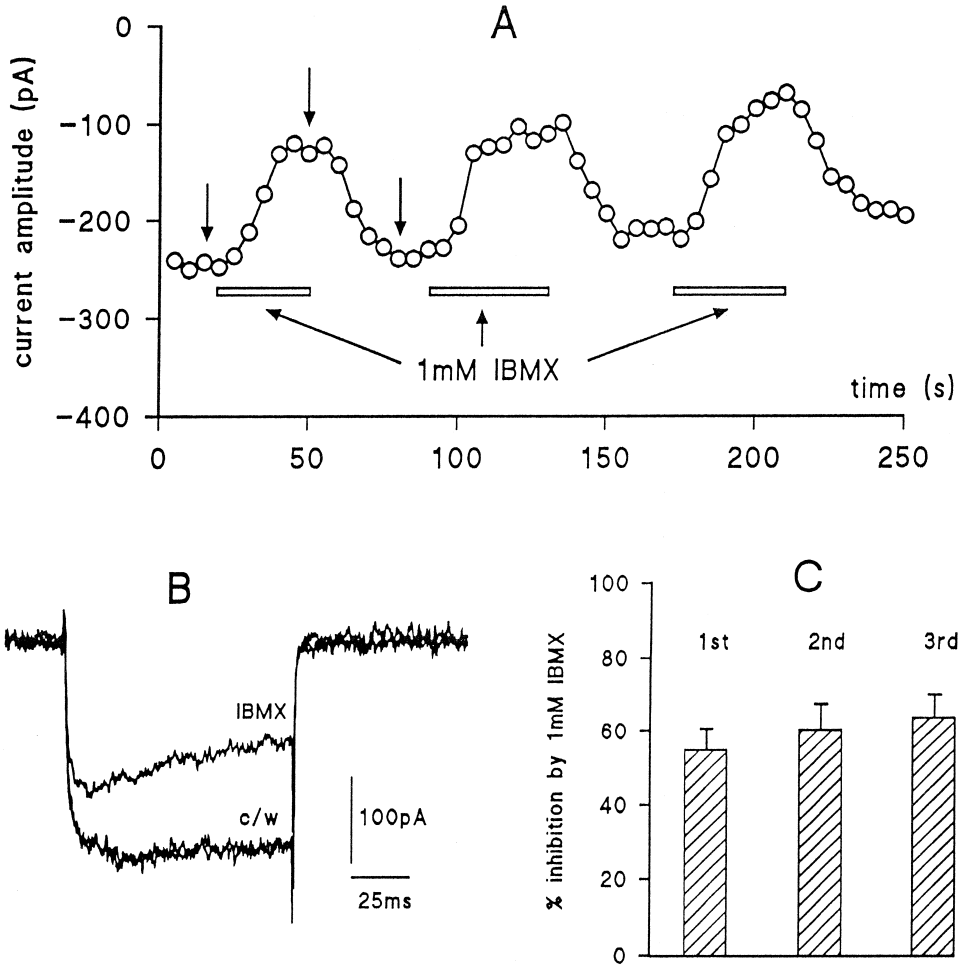

Fig. 1. (A) Time series plot of amplitudes of currents evoked by repeated step depolarizations from −80 mV to +10 mV. IBMX (1 mM) was applied as indicated by the horizontal bars. (B) Currents evoked during the experiment shown in (A), before (c), during (IBMX) and after (w) IBMX application. The corresponding measured amplitudes are arrowed in (A). (C) Bar graph showing the mean (with vertical S.E.M. bar) inhibitory effect of IBMX applied for the first, second and third time to cells ($n = 5$).

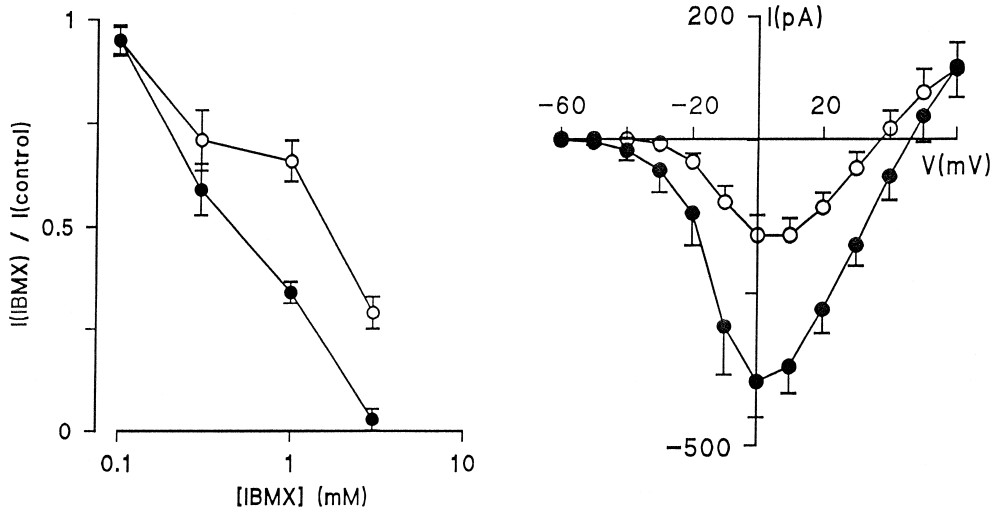

Fig. 2. (A) Concentration–response relationships for the effects of IBMX on Ca^{2+} channel currents. Each point plotted is the mean (±S.E.M., taken from between 5 and 9 cells in each case) inhibition of currents evoked by step depolarizations from −80 mV to +10 mV. Since currents evoked in the presence of IBMX were transient, their amplitudes were measured at their peaks (open symbols) and at the end of the depolarizing steps (filled symbols). In the absence of IBMX, currents showed little or no inactivation and amplitudes were measured at the end of depolarizing steps. (B) Mean (±S.E.M., $n = 6$) current–voltage relationships obtained before (filled circles) and during (open circles) bath application of 1 mM IBMX.

NaCl 95, CsCl 5, $MgCl_2$ 0.6, $BaCl_2$ 20, HEPES 5, D-glucose 10, tetraethylammonium-Cl 20 (pH 7.4, 21–24°C). IBMX was dissolved directly into the perfusate using sonication to facilitate dissolution. Experiments were performed at room temperature (21–24°C). Cells were voltage-clamped at −80 mV and step depolarized to various test potentials for 100 ms (0.1 Hz).

Evoked currents were filtered at 1 kHz, digitised at 2 kHz and stored on computer for measurement of current amplitude. In the absence of IBMX, amplitudes were measured over the last 10–15 ms of each step depolarization since they displayed little or no inactivation during step depolarizations. When IBMX was applied (see e.g. Fig. 1B) inactivation was observed and currents were measured at their peak as well as over the last 10–15 ms of each step. Except where stated, calculations of effects of drugs are based on measurements of current amplitudes over the last 10–15 ms of each step. All analysis and voltage protocols were performed using an Axopatch 200 A amplifier in combination with a Digidata 1200 and PClamp 6.0.2 software (Axon Instruments).

3. Results

All data presented here were obtained from a clonal line of HEK 293 cells in which the human cardiac L-type Ca^{2+} channel α_{1C} subunit was stably expressed. These currents were increased in amplitude by bath application of Bay K 8644 (2 μM) and fully inhibited by nifedipine (2 μM), indicating that all currents arose from activation of recombinant L-type Ca^{2+} channel subunits (Fearon et al., 1997).

Fig. 1A illustrates the inhibitory action of bath applied IBMX (1 mM). Whilst repeated step depolarizations to +10 mV (holding potential −80 mV) were applied to evoke currents, the perfusate was switched to one containing 1 mM IBMX and this always caused a rapid, reversible inhibition of current amplitude. Typical traces obtained before, during and after IBMX application are illustrated in Fig. 1B. Note that in the presence of IBMX, currents were reduced in amplitude and displayed pronounced inactivation. Repeated applications (up to 3 times) of IBMX to the same cell always caused inhibiton (e.g. Fig. 1A), and the degree of inhibition did not decline: mean inhibitions caused by three successive applications of IBMX are shown in Fig. 1C for 5 cells tested.

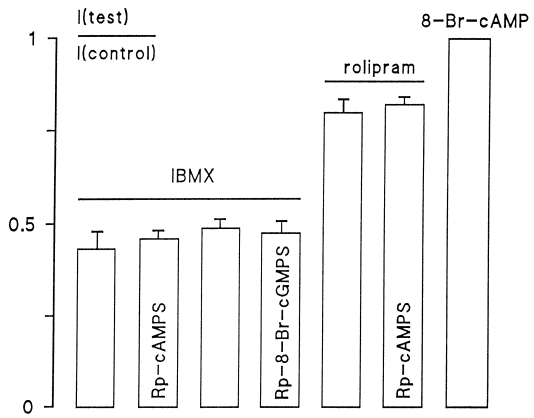

Fig. 3. Bar graph showing the mean (±S.E.M., $n = 4–5$ cells for each bar) degree of current inhibition caused by IBMX, rolipram and 8-Br-cAMP, as indicated. All three compounds were bath applied. The effects of IBMX and rolipram were tested in alternate cells which were intracellularly dialysed using patch pipettes filled with and without the protein kinase A inhibitor Rp-cAMPS (400 μM) or the protein kinase G inhibitor Rp-8-Br-cGMPS (50 μM), as indicated (empty bars indicate that no cyclic nucleotide analogue was included in the pipette).

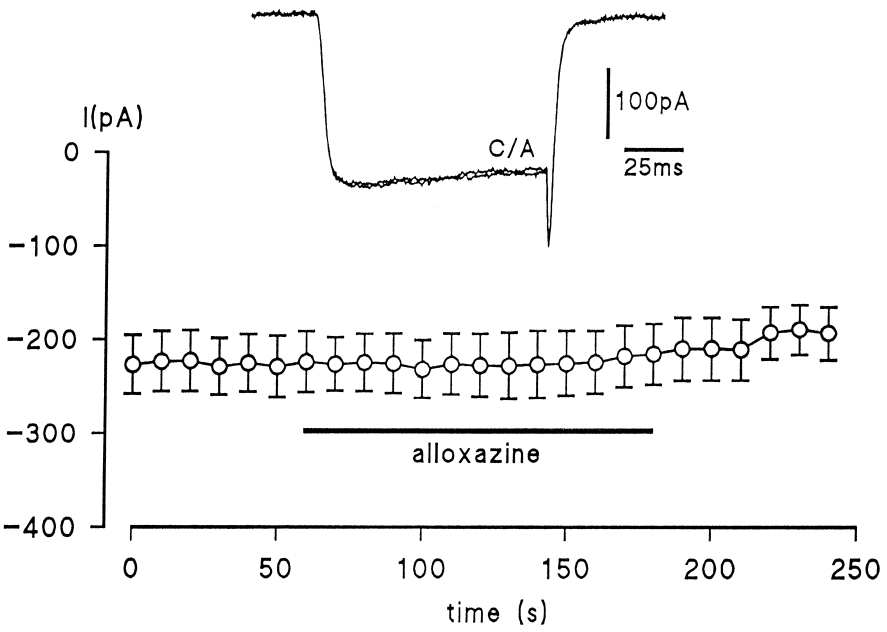

Fig. 4. Mean (±S.E.M., $n = 8$ cells) time series plot of current amplitudes evoked by successive step depolarizations from −80 mV to +10 mV. The non-selective, non-xanthine adenosine antagonist alloxazine (50 μM) was applied for the period indicated by the bar. Inset, example currents evoked in an example cell before (C) and during (A) alloxazine application.

The concentration–response relationship for the inhibitory actions of IBMX on Ca^{2+} channel currents is plotted in Fig. 2A. Small (ca. 10%) inhibitory effects were observed at 0.1 mM, and at 3 mM currents were almost completely inhibited (as measured at the end of step depolarizations). Fig. 2B plots mean current–voltage relationships in the absence and presence of 1 mM IBMX. At all activating test potentials studied, IBMX caused inhibition of current amplitude and these inhibitions were of similar magnitude, indicating that inhibition was not a voltage-dependent phenomenon. The apparent shift in reversal potential is likely due to a background outward current, since when currents are fully blocked (e.g. with Cd^{2+}), the leak conductance is outwardly rectifying (not shown).

To investigate possible mechanisms underlying the actions of IBMX, we first examined whether its actions were altered by cyclic nucleotide inhibitors. The inhibitory effects of 1 mM IBMX were tested in cells alternately dialysed for at least 10 min with or without the protein kinase A inhibitor Rp-cAMPS (400 μM), or the protein kinase G inhibitor Rp-8-Br-cGMPS (50 μM). As indicated in Fig. 3, the presence of these protein kinase inhibitors was without effect on the ability of IBMX to inhibit Ca^{2+} channel currents. We also investigated the effects of the non-xanthine phosphodiesterase inhibitor rolipram (100 μM). We found this inhibitor to cause small reductions in current amplitudes, but this effect was also unaltered by dialysis of cells with 400 μM Rp-cAMPS (Fig. 3). Thus the actions of IBMX appeared to be independent of changes in intracellular cyclic nucleotide levels and indeed, under our recording conditions, bath application of 5 mM 8-Br-cAMP was without effect on current amplitudes (Fig. 3).

IBMX is also known to act as an adenosine receptor antagonist (Daly et al., 1981; Dolphin et al., 1986; Cuttler et al., 1993). To investigate whether this action could account for its ability to inhibit Ca^{2+} channel currents, we tested the effects of another non-selective antagonist, alloxazine. As illustrated in Fig. 4, alloxazine (50 μM) was without effect on Ca^{2+} channel currents when applied for 2 min. Furthermore, in the presence of alloxazine (50 μM), IBMX (1 mM) reduced current amplitudes to 31.3 ± 6.8% ($n = 8$), a value not significantly different from that seen in the absence of alloxazine.

4. Discussion

The use of IBMX as a phosphodiesterase inhibitor is well documented, and workers frequently use this compound in the millimolar concentration range (e.g. Cuttler et al., 1993; Hall et al., 1995; Levesque and Hume, 1995). In the present study, we have demonstrated that IBMX causes a reversible, concentration-dependent inhibition of currents evoked by activation of recombinant human cardiac L-type Ca^{2+} channel α_{1C} subunits stably expressed in HEK 293 cells. Current inhibition was voltage- and use-independent and was associated with an increased apparent inactivation, such that current amplitude decreased markedly in the presence of IBMX during 100 ms step depolarizations.

The effects of IBMX cannot be attributed to an inhibitory action on phosphodiesterases, since the degree of inhibition was unaffected by inhibitors of PKA and PKG

applied intracellularly via dialysis from the patch pipette (Fig. 3). Furthermore, although another non-xanthine phosphodiesterase inhibitor, rolipram, had small inhibitory actions, these were also unaffected by a PKA inhibitor. Finally, elevation of intracellular cAMP (by bath application of 5 mM 8-Br-cAMP) was without effect on current amplitudes.

Our results cannot be accounted for by an action of IBMX to release Ca^{2+} from intracellular stores, since the Ca^{2+} chelator EGTA was present at a high concentration intracellularly, and the inhibitory actions of IBMX were sustained as long as the drug was present. Furthermore, IBMX could be repeatedly applied to the same cell without loss of the degree of inhibition (Fig. 1A). This would not be the case if IBMX discharged intracellular Ca^{2+} stores, since we used Ba^{2+} as charge carrier in the present studies, and stores would have no means of re-filling in the absence of extracellular Ca^{2+}. Experiments reporting a lack of effect of the non-selective adenosine antagonist alloxazine (Fig. 4) strongly suggest that IBMX is not acting as an adenosine antagonist, preventing possible native receptor activation via release of endogenous adenine nucleotides. Furthermore, the ability of IBMX to inhibit currents was unaffected by the presence of alloxazine.

Our findings point to the most likely mechanism of action of IBMX as being a direct one, in which IBMX somehow interacts with the α_{1C} subunit to cause a reduction in current amplitude and accelerated inactivation. A similar direct effect was demonstrated for IBMX and caffeine on native L-type Ca^{2+} channels of vascular smooth muscle cells (Hughes et al., 1990) and sustained (presumed L-type) currents in GH_3 cells (Simasko and Yan, 1993). In these cells, both phosphodiesterase inhibitors caused voltage-independent inhibition of currents, and an increase in the rate of apparent current inactivation was reported (Simasko and Yan, 1993). These studies also discounted the involvement of intracellular Ca^{2+} stores (Hughes et al., 1990) and cAMP (Simasko and Yan, 1993). Together with the present study, this would indicate that IBMX (and presumably caffeine) can inhibit L-type Ca^{2+} channels via direct interaction with the pore-forming α_1 subunit. IBMX could act as an open channel blocker, since in its presence, the initial rate of activation appeared unaffected (see e.g. Fig. 1B), but as the channels open, they become blocked and so currents do not reach control amplitudes. Blockade could then continue during the step depolarization, so that increasing numbers of channels become blocked, giving rise to the decline in amplitude throughout the rest of the depolarizing steps. At present, such a mechanism cannot be proved or discounted, and indeed would imply a voltage-dependent action of IBMX. Such a conclusion would contrast with the lack of obvious voltage-dependence shown in Fig. 2, where IBMX caused a similar degree of inhibition at all activating test potentials studied. Most importantly, however, the overall inhibitory nature of IBMX on L-type Ca^{2+} channel subunits should be taken into consideration when xanthine phosphodiesterase inhibitors are employed in functional studies concerning channel phosphorylation.

Acknowledgements

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