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Inhibition of ATP-induced surfactant exocytosis by dihydropyridine (DHP) derivatives: a non-stereospecific, photoactivated effect and independent of L-type Ca^{2+} channels

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Abstract

Purinergic stimulation of surfactant secretion via exocytosis of lamellar bodies is mediated by an elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). We tested the dihydropyridine (DHP) analogues isradipine (+/-enantiomers), nifedipine and Bay K 8644 (racemic forms) on ATP-induced surfactant secretion and $[Ca^{2+}]_i$ in single type II cells, using FM1-43 and fura-2 fluorescence. None of the DHPs (2 μM) had an effect on ATP-induced surfactant secretion in the dark. They did, however, inhibit secretion in a concentration-dependent manner during illumination, particularly with UV light. This effect was not stereospecific, because it was mimicked by (−)-isradipine. In addition, (+)- or (−)-isradipine, but not nifedipine or Bay K 8644, elicited a slow increase of $[Ca^{2+}]_i$ during illumination with UV light, which was reversible by exposure to dark. None of the DHPs inhibited the ATP-induced Ca^{2+} signal. In perforated patch clamp experiments, depolarizing voltage steps did not induce L-type Ca^{2+} (Sr^{2+}) currents, even in the presence of the agonist Bay K 8644 (1 μM). We conclude that impairment of ATP-induced surfactant secretion by all tested DHPs and alterations of Ca^{2+} homeostasis by isradipine are photoactivated effects, independent of L-type Ca^{2+} channels. © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Isradipine; Surfactant; Exocytosis; L-type Ca^{2+} channel; Lung; Alveolus

1. Introduction

A key step in the course of surfactant secretion by alveolar type II cells is exocytosis of lamellar bodies (reviewed in [1–5]). It is controlled by various physical and chemical factors, one of which being an elevation of $[Ca^{2+}]_i$ [6,7]. Recently, we determined a very low threshold $[Ca^{2+}]_i$ for lamellar body exocytosis in the range of 300 to 400 nM [8]. In contrast to other secretory cell types, type II cells exhibit slow secretion, and Ca^{2+} triggers exocytosis with a delay of seconds to minutes [8–10].

Among the various ways to elevate $[Ca^{2+}]_i$ and to stimulate surfactant secretion, ATP is one of the most potent agonists [11]. Several steps within the signaling pathway of ATP have been well characterized, including the release of Ca^{2+} from inositol 1,4,5-trisphosphate ($InsP_3$)-sensitive Ca^{2+} stores [12,13]. Although a large body of evidence suggests a physiological role of intracellular Ca^{2+} release during ATP-induced surfactant secretion, the existence and function of Ca^{2+} channels in the plasma membrane are still poorly addressed issues. Nevertheless, recent pharmacological evidence supports a role of L-type Ca^{2+} channels for surfactant secretion: Sen *et al*. reported inhibition by the L-type Ca^{2+} channel blockers nifedipine and nitrendipine and activation by the L-type Ca^{2+} channel agonist Bay K 8644 [14,15]. In addition, verapamil was found to inhibit Ca^{2+} influx into type II cells, although the effect on secretion was multiphasic [16]. Finally, in the alveolar epithelial cell line L2, we reported ATP-induced transient inward currents via L-type Ca^{2+} channels and high-affinity dihydropyridine receptors stereoselectively labeled by (+)-[^{3}H]-isradipine [17].

Hence, the aim of the present study was to elucidate the role of L-type Ca^{2+} channels in isolated type II cells, studied less than one day after preparation from rat lung. We

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Abbreviations: $[Ca^{2+}]_i$, cytoplasmic Ca^{2+} concentration; DHP, dihydropyridine; Bay K 8644, 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]pyridine-3-carboxylic acid methyl ester.

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found no evidence for the existence of these channels. However, we encountered yet unknown, photoactivated effects of DHPs.

2. Materials and methods

2.1. Type II cell preparation

This was done according to the method of Dobbs [18] with minor modifications as recently described [10]. At the end of the cell preparation from adult male Sprague-Dawley rats weighing about 200 g, type II cells were suspended in DMEM supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/ml streptomycin and 24 mmol/L $NaHCO_3$ and seeded on glass cover slips at low density ($\approx$40 cells per mm^2). Cells were given 3–48 hr time to settle in a 95% humidified air plus 5% CO_2 (37°) until use for experiments.

2.2. Perforated patch clamp experiments

Perforated patch clamp measurements of the whole cell current were done as recently described [19]. The control bath solution contained (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 glucose, 10 HEPES, pH 7.4. The "Sr^{2+}-solution" contained (in mM) 10 $SrCl_2$, 140 NaCl, 1 $MgCl_2$, 5 glucose, 10 HEPES. The control pipette solution contained 138 mM potassium gluconate, 1 mM $MgCl_2$, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 mM HEPES, and 300 μg/mL amphotericin B (freshly prepared every day from a stock solution of 60 mg/ml in dimethyl sulfoxide), pH 7.3. Ca^{2+} (Sr^{2+}) currents were measured as described in [17]. In short, voltage steps to between -40 mV and $+95$ mV were performed from a holding potential of -80 mV in 15-mV increments. Leak subtraction was performed online by use of six depolarizing subpulses before each voltage step. The currents were measured with an EPC7 amplifier and stored at a sampling rate of 1 kHz on a PC.

2.3. Simultaneous measurements of surfactant secretion and the fura-2 ratio

Determination of surfactant secretion by FM1-43 fluorescence was recently described in detail in [9] and [10]. This method is based on the cell-impermeant, surfactant-staining, properties of FM1-43, resulting in localized fluorescence after exocytosis, when FM1-43 enters lamellar body contents from the bath solution through the fusion pore. Importantly, FM1-43 is non-fluorescent in aqueous solutions, enabling measurements in the continuous presence of the dye (1 μM) in the bath. FM1-43 fluorescence intensity is proportional to the amount of exocytosed material, because surfactant does not readily dissolve in aqueous solutions [9]. Note that FM1-43 fluorescence is a parameter for vesicle fusion with the plasma membrane (which is almost complete after 20 min) and *not* for the release of secreted material through fusion pores into the extracellular space. Vesicle fusion with the plasma membrane can also be assessed by counting brightly-stained fluorescent spots instead of measuring FM1-43 fluorescence intensity [10], if a 2-D-imaging system with high magnification is used. Each fluorescent spot represents a location of vesicle fusion. Since compound exocytosis occurs in type II cells and more than one vesicle may fuse with the plasma membrane at a certain location, the number of fluorescent spots may underestimate the number of fused vesicles [9]. In this study, exocytic responses were assessed both by counting fluorescent spots (imaging system) and by measuring the FM1-43 fluorescence (photomultiplier), either alone or in conjunction with the fura-2 ratio, using an inverted microscope equipped with a photomultiplier or a 2-D-imaging system, as described [10]. At each excitation wavelength (340 nm and 380 nm for fura-2, 480 nm for FM1-43), cells were illuminated for between 20 and 120 ms (depending on the experimental system used), at a rate of 1 Hz.

2.4. Materials and statistics

Drugs were purchased from SIGMA. (+)- and (−)-isradipine were kindly provided by J. Striessnig, Innsbruck. Data are reported as means ± standard error.

3. Results

3.1. Lack of L-type Ca^{2+} (Sr^{2+}) currents in alveolar type II cells

Although several patch clamp investigations on type II cells have been reported, a definite search for L-type Ca^{2+} currents has not yet been performed. Therefore, we aimed at functional evidence in favor or against expression of these channels. Among all cells under study (N = 53), none of them exhibited a measurable voltage-gated inward current in response to depolarizing voltage steps. Hence, native type II cells are clearly different from the alveolar cell line L2, where L-type Ca^{2+} (or Sr^{2+}) currents are readily seen using conventional whole cell [17] or perforated [20] patch clamp methods. In order to maximize potential L-type currents, Sr^{2+} instead of Ca^{2+} was used as the charge carrier (N = 43), and the Ca^{2+} channel agonist Bay K 8644 (1 μM) was added to the bath solution (N = 33). Although voltage-gated outward currents were constantly observed at potentials more positive than ≈ -10 mV, inward currents were absent. Since protein kinase A may activate L-type Ca^{2+} channels, and isoproterenol increases cAMP levels in type II cells [21], experiments using Sr^{2+} and Bay K 8644 were also done in the presence of 10 μM isoproterenol (N = 11). Activation of inward currents, however, did not occur.

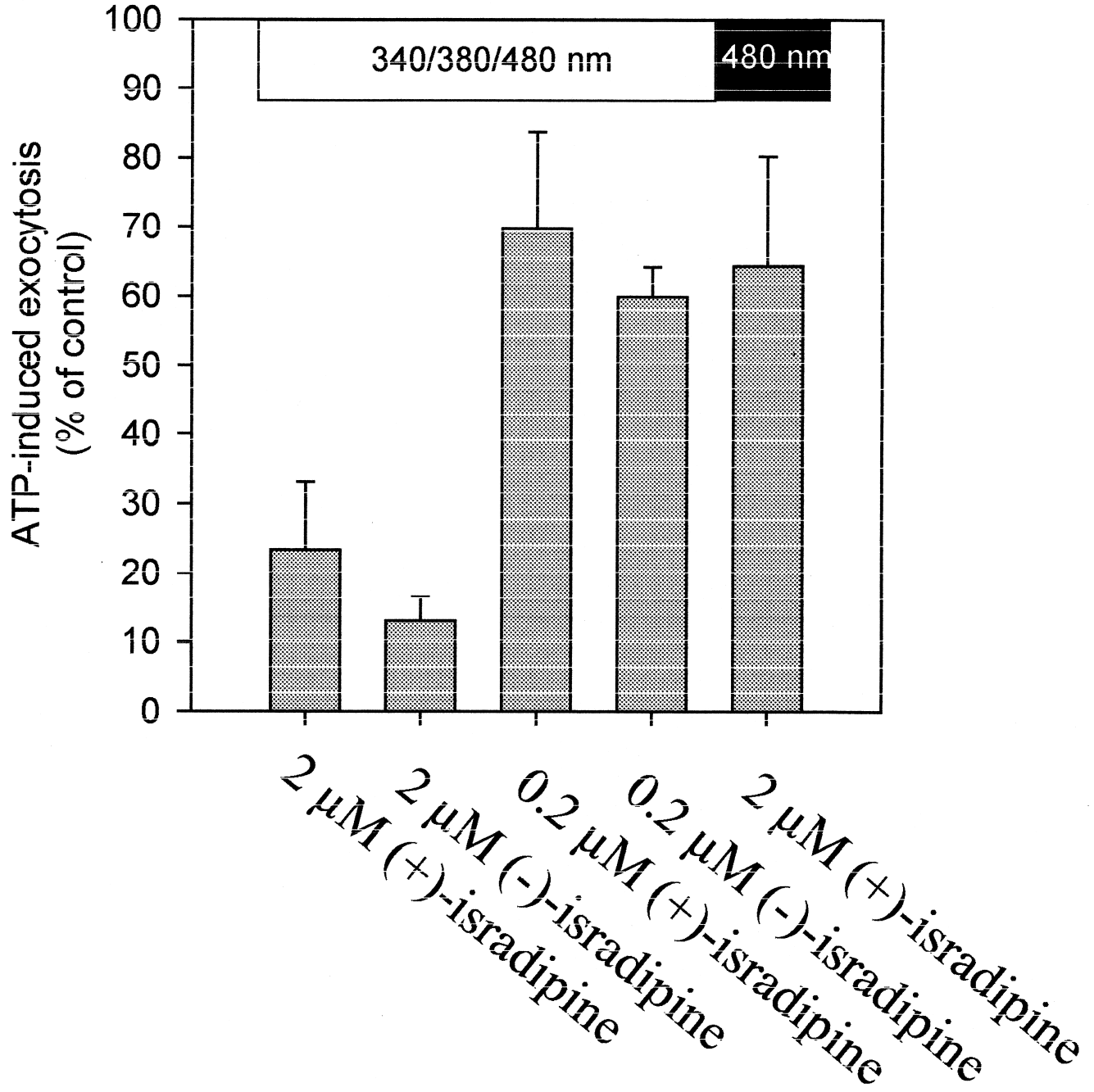

Fig. 1. Inhibition of ATP-induced exocytosis by isradipine during illumination is dose-dependent, not stereospecific and depends on the wavelength of light. ATP (10 μM)-induced exocytosis was determined during continuous illuminations at wavelengths indicated above bars (20 ms at each wavelength at a rate of 1 Hz). Control is the ATP-induced exocytic response in the absence of blocker and defined as 100%. To compensate for differences between cell preparations, each blocker experiment was preceeded by its own control experiment. In all experiments, the exocytic response was continuously assessed by measuring FM1-43 fluorescence (see section 2). In this set of experiments, the exocytotic response was quantified by measuring the difference in FM1-43 fluorescence intensity before and 20 min after addition of drugs and by correcting (dividing) this signal for the area of the studied cell monolayer. See section 2 for further details on experimental protocols. No apparent difference in the time course of fusion between the control and the experimental groups was found (data not shown). In the experimental groups, (+)- or (−)-isradipine was applied simultaneously with ATP. Each experimental group comprises ≥6 independent experiments, each of which contained between 5 and 20 cells.

3.2. Light-dependent, non-stereospecific inhibition of secretion by DHP derivatives

Despite this lack of L-type Ca^{2+} currents, (+)-isradipine, a stereoselective, high-affinity L-type Ca^{2+} channel blocker (reviewed in [22]) inhibited ATP-induced exocytosis of lamellar bodies in a concentration-dependent manner (Fig. 1). This effect was, however, non-stereospecific, be-

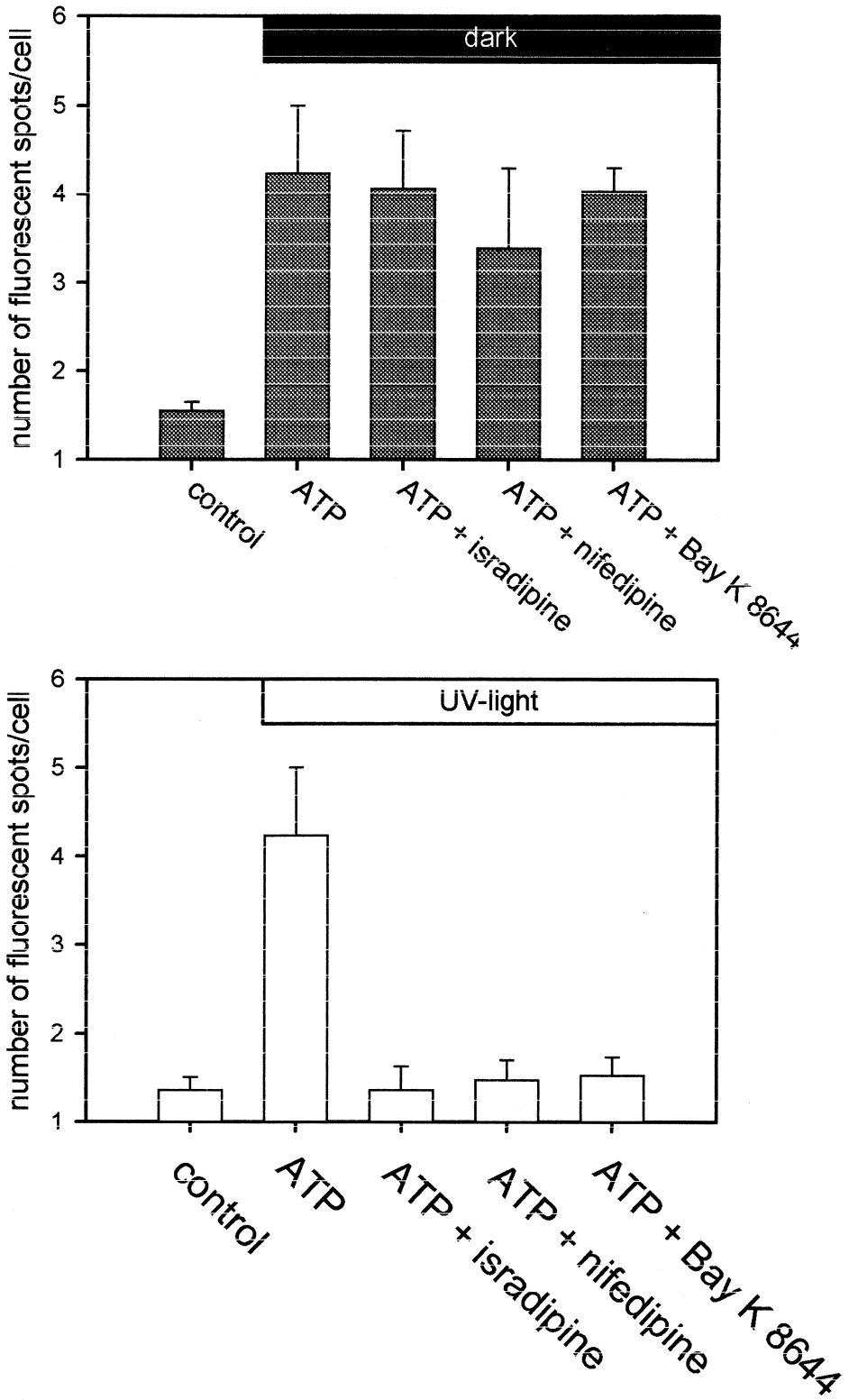

Fig. 2. Inhibition of exocytosis by DHP derivatives isradipine, nifedipine and Bay K 8644 is absent in the dark. Number of brightly-stained spots per cell appearing within 20 min of stimulation. Each brightly-stained spot in the continuous presence of 1 μM FM1-43 in the bath solution corresponds to the fusion between plasma membrane and vesicles. Spots were counted before and after the experimental period (20 min) and divided by the number of cells under study. In the dark experiments, illumination was switched off shortly before addition of drugs (10 μM ATP and 2 μM DHPs) and was turned on again 20 min later for counting. Except for (+)-isradipine, the racemic forms were used. Each bar represents pooled data from at least 3 independent experiments. Control indicates a 20 min observation period of untreated cells ("constitutive exocytosis"). Isradipine was given as (+) enantiomer, nifedipine and Bay K 8644 in their racemic forms. The effects of ATP plus drugs were not significantly different from ATP alone in the dark.

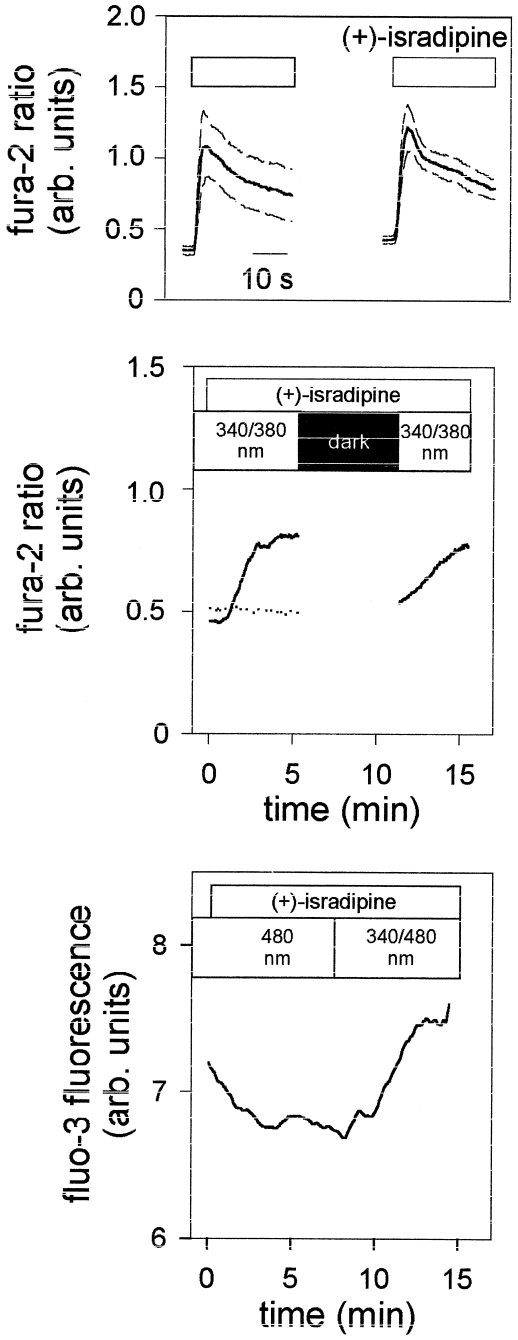

Fig. 3. Isradipine does not inhibit ATP-induced Ca^{2+} mobilization but slowly elevates $[Ca^{2+}]_i$ during illumination. Upper graph: ATP (10 μM)-induced rise of the fura-2 ratio (full lines, means; dashed lines, SEMs) in the absence (control) or presence of 2 μM (+)-isradipine. In these experiments, (+)-isradipine was added in the dark to avoid photoactivated effects, and ATP was added immediately after turning on illumination (340/380/480 nm). Middle graph: Representative examples of the fura-2 ratios of individual type II cells in response to illumination, studied in the presence (solid line) or absence (dotted line) of Ca^{2+} (absence of Ca^{2+} indicates a Ca^{2+}-free, 1 mM EGTA-containing bath solution and a 15 min pretreatment with 10 μM BAPTA-AM). 2 μM (+)-isradipine was added during repetitive illuminations at 340/380 nm (for 120 ms at each wavelength at a rate of 1 Hz). Evidently, the fura-2 ratio could not be measured when illumination was turned off (dark). Lower graph: The fluo-3 fluorescence of an individual type II cell in the presence of 2 μM (+)-isradipine. During illumination at 480 nm, photobleaching (i.e. a decrease) of fluorescence is observed. During alternative illuminations at 340 nm and 480 nm, fluorescence was measured during exposure to 480 nm exclusively. (−)-isradipine exerted the same effects (data not shown).

cause it was elicited by (−)-isradipine with the same concentration-dependence as with the (+)-enantiomer (Fig. 1). The lack of stereospecificity indicates that the action of isradipine is independent of its high affinity receptor site on the α-subunit of the Ca^{2+} channel [23], and this prompted us to look for alternative mechanisms. We found that the inhibition of exocytosis by (+)-isradipine was strongly dependent on the wavelength of light at which the cells were exposed during the experiment (illuminations at 340 nm, 380 nm [fura-2] and 480 nm [FM1-43]) and pronounced (though not exclusively elicited) in the UV range (Fig. 1).

Light dependency of the effect of (+)-isradipine is further proven by the lack of inhibition in the absence of light. Fig. 2 reveals that in the dark, (+)-isradipine did not significantly inhibit secretion at the same concentration as above (2 μM). This light-dependent effect was not only confined to isradipine, but also observed with the DHP derivatives nifedipine and Bay K 8644 (we used the racemic forms of nifedipine and Bay K 8644 at the same concentrations as isradipine, Fig. 2). The exocytotic response was assessed 20 min following addition of ATP (10 μM), a period after which the majority of the exocytotic response was found to be completed [10].

3.3. Effects of DHP derivatives on $[Ca^{2+}]_i$

We tested if photoactivated inhibition of ATP-induced exocytosis by isradipine might be caused by impeded Ca^{2+} mobilization in response to ATP. Ca^{2+} mobilization was, however, unaffected by (+)-isradipine (when given prior to ATP in the dark) (Fig. 3). The channel blocker did, however, slowly increase the fura-2 ratio by itself, which was strictly dependent on illumination (Fig. 3). It was slowly reversible upon switching off illumination and exposing cells to dark (Fig. 3). The most plausible explanation for these observations is that illumination creates a short-lived, toxic derivative of (+)-isradipine, which profoundly affects Ca^{2+} homeostasis. Accordingly, the increase of the fura-2 ratio was abolished when the $[Ca^{2+}]_i$ elevation was prevented by a Ca^{2+} free (1 mM EGTA) bath solution and by preloading cells for 15 min with the Ca^{2+} chelator BAPTA/AM (10 μM) (Fig. 3). Besides, this finding excludes that the apparent increase of $[Ca^{2+}]_i$ might be a result of a shift of the emission spectrum of fura-2 by illuminated (+)-isradipine or of the generation of a fluorescent derivative of (+)-isradipine (the latter can be also excluded because the fura-2 fluorescence did not change at its isosbestic wavelength). These results are supported by independent experiments using a different Ca^{2+} indicator: When fluo-3 (a non-radiometric Ca^{2+} indicator with an excitation wave-

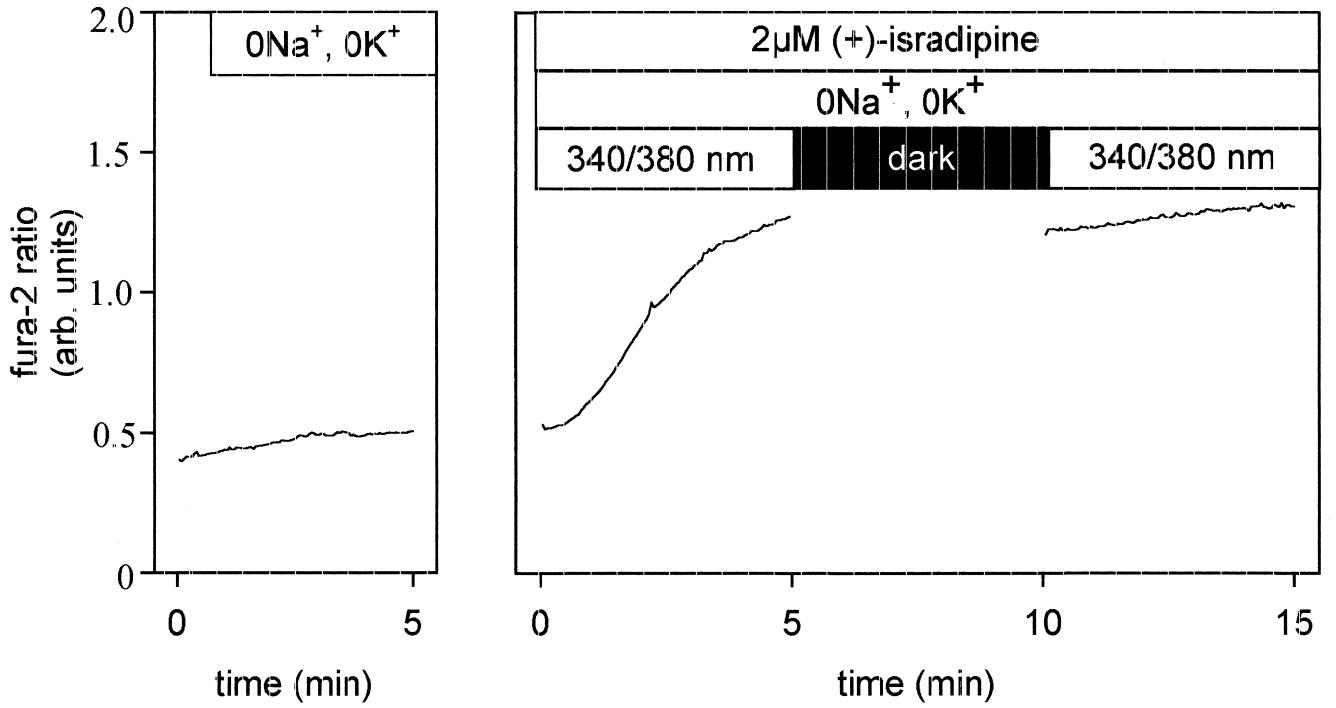

Fig. 4. Extracellular Na^+ and K^+ removal does not affect the isradipine-induced rise of $[Ca^{2+}]_i$. Both Na^+ and K^+ were replaced by D-methyl-N-glucamine$^+$. Illumination as in Fig. 3. Note that the recovery of $[Ca^{2+}]_i$ during dark is almost entirely blunted (compare with Fig. 3, middle graph).

length of 480 nm) was used, photobleaching occurred during illumination (Fig. 3). The bleaching of fluo-3 fluorescence was reversed by intermittend illuminations at 340 nm in the presence of (+)-isradipine, reflecting the rise of $[Ca^{2+}]_i$ (Fig. 3). The findings indicate that the photoactivated effect of isradipine is predominant in the UV range. This alteration in Ca^{2+} homeostasis was also non-stereospecific and mimicked by (−)-isradipine (data not shown).

Attempts were made to determine the origin and cellular mechanism of the effects of isradipine: Gd^{3+} (10–100 μM), a blocker of several non-voltage-gated cation and Ca^{2+} channels, did not prevent the UV-, (+)-isradipine-induced elevation of $[Ca^{2+}]_i$ (data not shown). This suggests that the photoactivated $[Ca^{2+}]_i$ elevation is not a result of an activation of store-operated or receptor-mediated Ca^{2+} channels. In order to elucidate a possible block of Na^+/Ca^{2+} exchange, we sought to gain insight into the role of this ion exchanger in type II cells by omitting Na^+ from the bath (replacement by N-methyl-D-glucamine$^+$), resulting in electrochemical gradients favoring Ca^{2+} uptake instead of extrusion. The results are shown in Fig. 4: Bath Na^+ removal elicited only a slow and small $[Ca^{2+}]_i$ elevation. Addition of (+)-isradipine, in the presence of UV light, caused a rapid $[Ca^{2+}]_i$ increase, which was not reversible after exposure to dark. This suggests that Na^+/Ca^{2+} exchange is not fundamental to maintain the resting $[Ca^{2+}]_i$ and therefore unlikely to mediate the isradipine-induced $[Ca^{2+}]_i$ rise. It has a role, however, in extruding Ca^{2+} when highly elevated. In summary, these experiments did not reveal evidence for a specific target (Ca^{2+} channel or exchanger) for photoactivated isradipine. The mechanism could be an unspecific "Ca^{2+} leak" or the inhibition of a plasmalemmal Ca^{2+} ATPase, either directly or by ATP depletion.

Other DHPs were investigated to test if isradipine-mediated effects on $[Ca^{2+}]_i$ are a general feature of DHP compounds. In contrast to the light-dependent inhibition of nifedipine and Bay K 8644 on surfactant exocytosis (see above), both drugs did not affect $[Ca^{2+}]_i$, neither at rest nor after stimulation with ATP (data not shown). This suggests that the effects of isradipine on Ca^{2+} homeostasis and surfactant secretion, respectively, are independent effects. Photoactivated elements of isradipine and nifedipine (or Bay K 8644) apparently differ in their potency to affect each of these processes. A structure-function-relation or the assumption of a putative photoactivated element cannot be inferred from these observations.

4. Discussion

The inhibition of secretion by DHPs shown here is an effect downstream the elevation of $[Ca^{2+}]_i$, since none of these drugs inhibited the ATP-induced Ca^{2+} release. We have shown in a recent study that selective $[Ca^{2+}]_i$ elevation (UV-flash photolysis of caged Ca^{2+}) can trigger exocytosis independent of receptor activation, indicating that a short Ca^{2+} signal by itself is a sufficient stimulus for secretion [8]. Ca^{2+}-sensitive elements for lamellar body fusion with

the plasma membrane, however, have not yet been identified.

Irrespective of what the photoactivated mechanism is, DHPs should be used with caution in illuminated systems, in particular in the presence of UV light. In our experiments, cells were always studied with UV light-transmitting objectives of high magnification (Zeiss 40× or 100× Fluar oil). Hence, the light intensity (from a 75 W Xenon lamp) was probably high in the focus plane, i.e. at the place where the cells were located, although usual for imaging systems and photometry. In other systems, light-induced effects of DHPs may play a minor role, depending on the light exposure time, the time course of the cellular function under study, the cell type and the blocker concentration. In fast processes such as synaptic transmission, these effects may be either non-existing or negligible when the time course of illumination is kept very short.

Our data rise the possibility that the observations by Sen et al. [14,15], who reported inhibition of endothelin-induced surfactant secretion by nifedipine and nitrendipine, may have also been the result of a photoactivated process. Although it was shown by these authors that the Bay K 8644-induced $[Ca^{2+}]_i$ elevation was sensitive to preincubation with nifedipine, this does not exclude unspecific DHP effects.

The lack of Ca^{2+} currents upon depolarization and the light-dependent, non-stereospecific actions of DHPs on surfactant secretion shown here are all clear arguments against the existence of L-type Ca^{2+} channels in type II cells. Since we studied cells within less than a day (a minimum of 3 hr) after isolation from rat lung, it is very unlikely that L-type Ca^{2+} channels were lost during dedifferentiation. Much more likely, these channels, which we had found in L2 cells [17], are a result of such de(trans?)differentiation. From a physiological standpoint, the lack of voltage-gated channels corresponds well with the slow time course of secretion.

Acknowledgments

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