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Biochemical Pharmacology 67 (2004) 1437–1444
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Fluorescence microscopy studies with a fluorescent glibenclamide derivative, a high-affinity blocker of pancreatic β-cell ATP-sensitive K^+ currents

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Received 31 July 2003; accepted 1 December 2003

Abstract

Hypoglycemic sulfonylureas (e.g. tolbutamide, glibenclamide) exert their stimulatory effects on pancreatic β-cells by closure of ATP-sensitive K^+ (K_{ATP}) channels. Pancreatic K_{ATP} channels are composed of two subunits, a pore-forming inwardly rectifying K^+ channel (Kir6.2) subunit and a regulatory subunit (the sulfonylurea receptor of subtype 1 (SUR1)) in a $(SUR1/Kir6.2)_4$ stoichiometry. The aim of the present study was to characterize the interaction of green-fluorescent 3-[3-(4,4 difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*S*-indacen-3-yl)propanamido] glibenclamide (Bodipy-glibenclamide) with pancreatic β-cell K_{ATP} channels using patch-clamp and fluorescence microscopy techniques. Bodipy-glibenclamide inhibited K_{ATP} currents from the clonal insulinoma cell line RINm5F half-maximally at a concentration of 0.6 nM. Using laser-scanning confocal microscopy Bodipy-glibenclamide was shown to induce a diffuse fluorescence across the RINm5F cell, but only about 17% of total Bodipy-glibenclamide-induced fluorescence intensity in RINm5F cells was due to specific binding to SUR1. Using fluorescence correlation spectroscopy, it could be demonstrated that the fluorescence label contributes to the protein binding and, therefore, possibly also to the non-specific binding of Bodipy-glibenclamide observed in RINm5F cells. Specific binding of Bodipy-glibenclamide to SUR1 in RINm5F cells might be localized to different intracellular structures (nuclear envelope, endoplasmic reticulum, Golgi compartment, insulin secretory granules) as well as to the plasma membrane. In conclusion, Bodipy-glibenclamide is a high-affinity blocker of pancreatic β-cell K_{ATP} currents and can be used for visualizing SUR1 in intact pancreatic β-cells, although non-specific binding must be taken into account in confocal microscopy experiments on intact β-cells.

Keywords: Pancreatic β-cell; K_{ATP} channel; Sulfonylurea receptor; Bodipy-glibenclamide; Laser-scanning confocal microscopy; Fluorescence correlation spectroscopy

1. Introduction

Hypoglycemic sulfonylureas (e.g. tolbutamide, glibenclamide) are widely used in the therapy of non-insulin-dependent diabetes mellitus. The hypoglycemic effects of sulfonylureas can be explained by a direct stimulation of the pancreatic β-cell *via* blockade of the K_{ATP} channel [1–4]. The K_{ATP} channel is an octameric 4:4 complex of a pore-forming inwardly rectifying K^+ channel (Kir6.2) subunit and a regulatory subunit, the SUR. Two different SUR genes have been cloned, SUR1 and SUR2, that show different tissue expressions and encode proteins with high (SUR1) and low (SUR2) sensitivity towards sulfonylureas, respectively. K_{ATP} channels in pancreatic β-cells comprise Kir6.2 and SUR1 subunits [5].

A fluorescently labeled sulfonylurea (Bodipy-glibenclamide; the chemical structure is shown in Fig. 1) has been used to study sulfonylurea binding to bovine monocytes [6] and to intact pancreatic β-cells [7], respectively. Recently, the Bodipy-glibenclamide labeling sites of freshly isolated mouse islet cells have been localized to insulin secretory granules which, therefore, have been identified as the major site of K_{ATP} channels of the endocrine pancreas [8]. The present study was performed in order to further characterize the interaction of Bodipy-glibenclamide with K_{ATP} channels from clonal insulinoma cell lines using

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Abbreviations: Bodipy-glibenclamide, 3-[3-(4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*S*-indacen-3-yl)propanamido] glibenclamide; K_{ATP} channel, ATP-sensitive K^+ channel; SUR, sulfonylurea receptor.

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doi:10.1016/j.bcp.2003.12.011

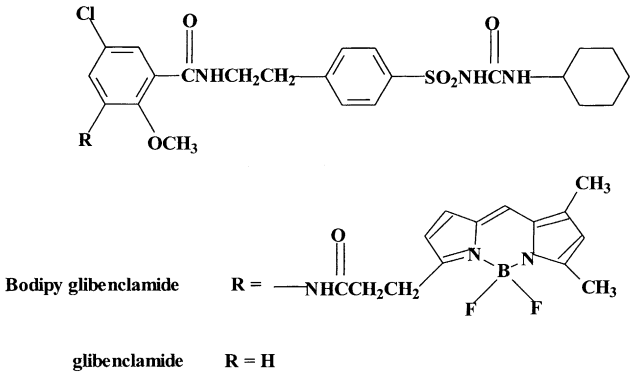

Fig. 1. Chemical structures of glibenclamide and of Bodipy-glibenclamide.

patch-clamp and fluorescence microscopic techniques (laser-scanning confocal microscopy and fluorescence correlation spectroscopy, FCS). It was the aim to differentiate between specific binding to SUR1 and non-specific binding of Bodipy-glibenclamide to RINm5F cells and to examine whether the fluorescence label contributes to the non-specific binding of Bodipy-glibenclamide.

2. Materials and methods

2.1. Culture of RINm5F cells

Cells from the clonal insulinoma cell line RINm5F were grown in RPMI 1640 medium (10 mM glucose), supplemented with 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 μg/mL) and 2 mM glutamine at 37° in a humidified atmosphere of 5% CO_2. Cells were passaged at 7-day intervals, plated onto 35 mm dishes and used 1–4 days after plating.

2.2. Binding of Bodipy-glibenclamide to microsomes from HIT-T15 cells

Binding experiments on HIT-T15 cells (SV-40 transformed hamster β-cells) were performed as described previously [9]. Briefly, the competition of varying concentrations of Bodipy-glibenclamide and of 0.3 nM [^{3}H]glibenclamide to resuspended HIT-T15 cell membranes was studied to calculate the K_D value (dissociation constant) for binding of Bodipy-glibenclamide to SUR1.

2.3. Electrophysiological recording

A standard patch-clamp technique was used in the whole-cell configuration [10]. A patch-clamp amplifier (EPC-7, List Electronic) was used. Pipette resistances ranged between 3 and 7 MΩ when filled with solution B. The membrane potential was held at −70 to −80 mV and hyper- and depolarizing voltage pulses of 10 mV amplitude and 200-ms duration were applied alternately every 2 s. Most of the current evoked by this pulse protocol is flowing through K_{ATP} channels [2]. Current signals were stored on a Sony PC204 DAT recorder, filtered at 2 kHz (−3 dB) with the help of a Bessel filter (902, Frequency Devices) and displayed on a digital oscilloscope (3091, Nicolet). Traces shown in the publication were plotted from the oscilloscope. Outward currents flowing from the cell interior to the bath are indicated by upward deflections and inward currents by downward deflections. Amplitudes of several successive current responses were read from the digital oscilloscope to measure the mean current accurately. Experiments were performed at room temperature (20–22°).

In order to investigate the effects of Bodipy-glibenclamide on K_{ATP} currents under steady-state conditions, cells were incubated for 1–2 hr in the extracellular solution A containing different concentrations of Bodipy-glibenclamide, and thereafter whole-cell currents were recorded. Differences in the cell size were taken into account by dividing the maximum current amplitudes to depolarizing pulses (10 mV) recorded from each cell by its membrane capacitance.

The concentration–response relationship for the inhibition of K_{ATP} currents by Bodipy-glibenclamide was calculated according to the Hill equation:

$$I = (I_{max} - a)\left(1 - \frac{A^n}{IC_{50}^n + A^n}\right) + a \quad (1)$$

where A = concentration of Bodipy-glibenclamide, n = slope parameter (Hill coefficient), IC_{50} = half-maximally inhibitory concentration of Bodipy-glibenclamide. I_{max} was the maximum current amplitude divided by the membrane capacitance under control conditions (absence of Bodipy-glibenclamide) and I was the maximum current amplitude divided by the membrane capacitance in the presence of Bodipy-glibenclamide. Constant a was included in order to describe currents different from K_{ATP} currents which are not sensitive to sulfonylureas.

2.4. Laser-scanning confocal microscopy on intact RINm5F cells in the presence of Bodipy-glibenclamide

For the measurement of the binding of Bodipy-glibenclamide to RINm5F cells, cells were grown on a coverglass and incubated for 1–2 hr in solution C containing both 10 nM green-fluorescent Bodipy-glibenclamide (absorbance maximum at 504 nm) and 300 μM tolbutamide. Thereafter, the fluorescence intensity of RINm5F cells was determined first before and then after washout of tolbutamide. An inverted Zeiss LSM-510 laser-scanning confocal imaging system with an argon laser (excitation wavelength: 488 nm) was employed. Cells were illuminated through a water immersion objective lens (C-Apochromat 40×/1.2). An excitation dichroic mirror with a cutoff of 488 nm and a long-pass emission filter with a cutoff of 505 nm were used to detect Bodipy-glibenclamide-induced fluorescence using a photomultiplier tube.

3.9 s were required for scanning an image area of 512× 512 pixels corresponding to 5800 μm^2. Optical sections with a diameter of 1 μm across the axial (z) axis were scanned through the middle of the cells at time intervals of 60 s. Intensity values for each cell were obtained by calculating the average brightness values of each optical section measured on an arbitrary gray scale from 0 (blackest) to 255 (whitest). In all experiments confocal microscope settings were maintained constant. Images were reproduced with a Sony UP-D1510CNE Digital Color Printer. Laser-scanning confocal microscopy experiments were performed at room temperature (20–22°).

The increase of the Bodipy-glibenclamide-induced fluorescence intensity after washout of tolbutamide was calculated according to the equation:

$$100\left(\frac{F}{F_{\text{tolb}}} - 1\right) = C(1 - \mathrm{e}^{-(t-b)/\tau_{\text{app}}}) \quad (2)$$

where F was the Bodipy-glibenclamide-induced fluorescence intensity at different times (t) after washout of tolbutamide, F_{tolb} was the Bodipy-glibenclamide-induced fluorescence intensity in the simultaneous presence of tolbutamide directly before washout of tolbutamide, b was the time for exchange of the bath solution, τ_{app} was the time constant for the Bodipy-glibenclamide-induced increase of fluorescence intensity after washout of tolbutamide, and C was defined as $100(F_{\text{max}}/F_{\text{tolb}} - 1)$, where F_{max} was the calculated maximum asymptotic value of the Bodipy-glibenclamide-induced fluorescence intensity after washout of tolbutamide.

2.5. Fluorescence correlation spectroscopy

FCS records the thermodynamic fluctuations of fluorescently labeled molecules after excitation by a focused laser beam in a small volume element of below 1 fl [11]. FCS measurements were performed with a ConfoCor 2 fluorescence correlation spectrometer (Carl Zeiss Jena). The samples were excited using an argon laser (excitation wavelength: 488 nm) and fluorescence emission was detected at wavelengths of >505 nm.

For the determination of the binding of Bodipy-glibenclamide to albumin, Bodipy-glibenclamide at a concentration of 10 nM was incubated with albumin concentrations of 10 and 100 μg/mL, respectively, at room temperature in distilled water for at least 1 hr. Some protein binding experiments at 100 μg/mL albumin were performed in the simultaneous presence of both 10 nM Bodipy-glibenclamide and 1 μM non-labeled glibenclamide. Fifty microliters of a sample solution was added to a coverglass, which was placed above the objective lens (C-Apochromat 40×/1.2 water immersion) through which the laser beam passed, and the same objective lens served for the collection of the fluorescence emission. Spectra were recorded for 30 s; each single measurement was repeated 10 times, and the averaged results are shown in Figs. 4 and 5. The species counted by FCS are free Bodipy-glibenclamide and Bodipy-glibenclamide bound to albumin. The intensity fluctuations were analyzed by an autocorrelation function ($G(t)$), as implemented in the program ConfoCor 2, using the following two-component model corresponding to free and albumin-bound Bodipy-glibenclamide:

$$G(t) = 1 + \frac{(1 - \mathrm{TA}(1 - \mathrm{e}^{-t/t_0}))}{\mathrm{N}(1 - \mathrm{TA})} \times \left(\frac{(1 - Y)}{(1 + t/\tau_1)} \times \frac{1}{(1 + 1/S^2(t/\tau_1))^{1/2}} + \frac{Y}{(1 + t/\tau_2)} \times \frac{1}{(1 + 1/S^2(t/\tau_2))^{1/2}} \right) \quad (3)$$

where TA is the average fraction of dye molecule in the triplet state with relaxation time t_0, N is the total average number of fluorescent molecules in the observation volume, Y is the relative concentration of Bodipy-glibenclamide bound to albumin, τ_1 and τ_2 define the average time (diffusion time constants) for detected molecules of free and bound Bodipy-glibenclamide, respectively, and S is the structure parameter defining the ratio between the axial (ω_z) and the lateral (ω_{xy}) half-axes of the observation volume ($S = \omega_z/\omega_{xy}$). ω_z and ω_{xy} are the axial and lateral distances, respectively, between the coordinate where the Gaussian emission light distribution adopts the maximum value and the point where the light intensity decreases down to 1/e^2 of the maximum value (observation volume).

The value of the diffusion coefficient for rhodamine 6G ($D = 2.8 \times 10^{-10}$ m^2/s) was used for determination of the parameters ω_{xy} and S prior to the experiment. The diffusion time constant is related to ω_{xy} through:

$$\tau_{\text{d}} = \frac{{\omega_{xy}}^2}{4D} \quad (4)$$

The structure parameter S and the detection volume evaluated by FCS were 10 and 0.4 fl, respectively. The structure parameter S obtained with rhodamine 6G was fixed in autocorrelation functions of further experiments.

2.6. Statistical analysis

Values in the text and figures are presented as mean values ± SEM.

2.7. Solutions and chemicals

The bath (solution A) in whole-cell patch-clamp experiments contained (concentrations in mM): 140 NaCl, 5.6 KCl, 1.2 MgCl, 2.6 $CaCl_2$ and 10 HEPES titrated to pH = 7.40 with NaOH. The pipette (solution B) in whole-cell patch-clamp experiments contained (mM): 140 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, 0.3 ATP, 5 HEPES titrated to pH = 7.15 with KOH. In laser-scanning confocal microscopy experiments on intact RINm5F cells

$CaCl_2$ was replaced by 1 mM EGTA in solution A in order to prevent sulfonylurea-induced increases of the cytosolic Ca^{2+} concentration (solution C). Bodipy-glibenclamide was purchased from Molecular Probes and glibenclamide was from Sigma. Stock solutions of 100 μM Bodipy-glibenclamide and 10 mM glibenclamide were prepared in DMSO and applied to solution A to give the final concentrations. BSA (fraction V, pH 7.0) was from Sigma.

3. Results

A maximum current density of 16.3 ± 1.1 pA/pF in response to 10 mV depolarizing pulses was found in 25 control experiments on RINm5F cells (in the absence of Bodipy-glibenclamide, Fig. 2), and a similar value of 17.6 pA/pF has been reported previously for mouse pancreatic β-cells [3]. Sulfonylureas (e.g. tolbutamide and glibenclamide) have previously been shown to concentration-dependently inhibit pancreatic β-cell K_{ATP} currents as evoked by this pulse protocol [2,3]. Exposure of the cells to Bodipy-glibenclamide caused a concentration-dependent decrease of the current density (Fig. 2A). However, even at high concentrations of sulfonylureas, small whole-cell currents were observed (2.7 ± 0.7 pA/pF ($N = 8$) in the presence of 30 nM Bodipy-glibenclamide (Fig. 2), 2.1 ± 0.2 pA/pF ($N = 25$) in the presence of 300 μM tolbutamide (not shown)). These currents might arise from

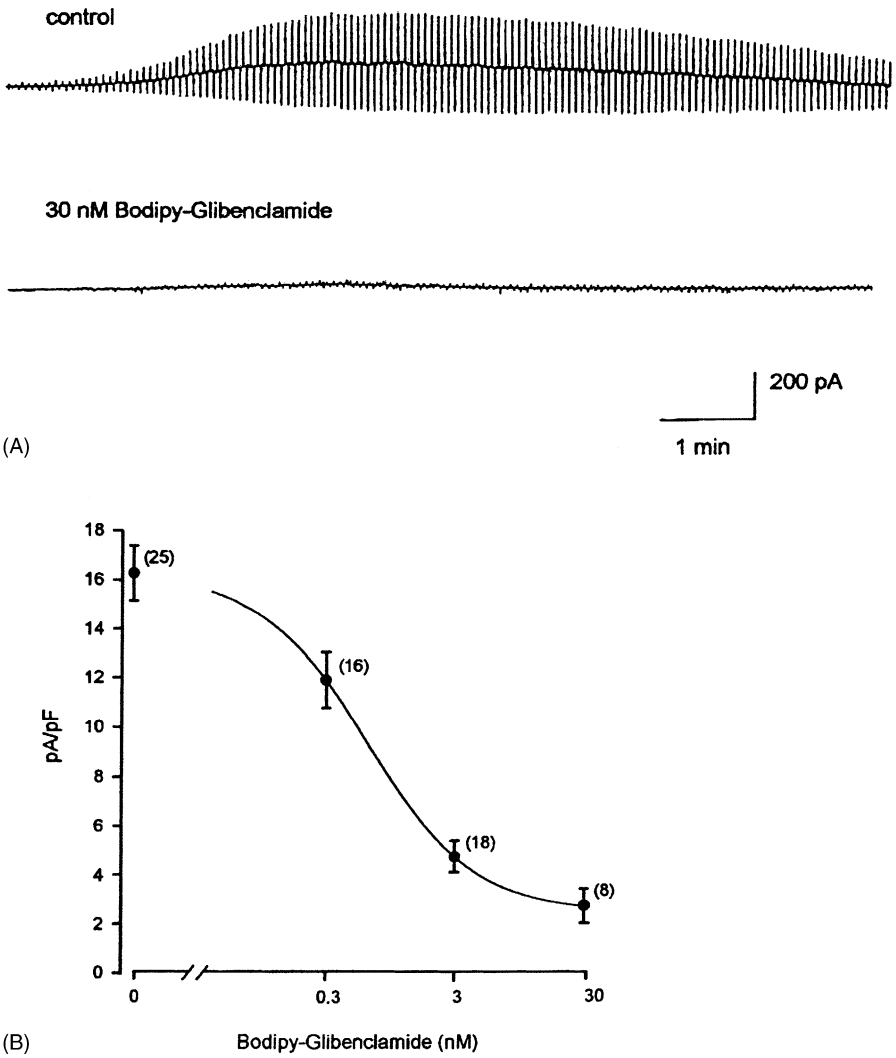

Fig. 2. Effects of Bodipy-glibenclamide on K_{ATP} currents from RINm5F cells. (A) Cells were incubated for 1–2 hr in the presence of different concentrations of Bodipy-glibenclamide, and thereafter membrane currents were measured. Current amplitudes under control conditions increased during the first minutes of recording due to the washout of ATP from the cytoplasm; after reaching a maximum, the currents decreased due to spontaneous run-down. Maximum currents in response to 10 mV depolarizing pulses were divided by the cell capacitance and were 20.4 pA/pF under control conditions and 1.5 pA/pF in the presence of 30 nM Bodipy-glibenclamide. (B) Concentration–response relationship for the inhibition of whole-cell K_{ATP} currents from RINm5F cells by Bodipy-glibenclamide. The ordinate represents current densities in the presence of different concentrations of Bodipy-glibenclamide. The abscissa indicates the Bodipy-glibenclamide concentrations (logarithmic scale). Points represent means and the vertical lines the SEM. Numbers of observations are given in parentheses. The line is a fit to Eq. (1).

currents different from K_{ATP} currents (e.g. leak currents between the pipette and cell membrane). Therefore, constant a was included in Eq. (1), and the value fitted for a was 2.5 ± 1.6 pA/pF. The curve in Fig. 2B resulted from a fit to Eq. (1) and gave a half-maximally inhibitory concentration of 0.6 ± 0.3 nM Bodipy-glibenclamide and a slope parameter n of 1.0 ± 0.3.

Bodipy-glibenclamide bound to microsomes from HIT-T15 cells with a K_D value of 1.7 nM.

In order to visualize SURs in cultured RINm5F cells, laser-scanning confocal images of RINm5F cells labeled with 10 nM Bodipy-glibenclamide were obtained (Fig. 3). In order to differentiate between specific binding to SUR1 and non-specific binding of Bodipy-glibenclamide to RINm5F cells, Bodipy-glibenclamide-induced fluorescence was determined first in the presence and then after washout of 300 μM tolbutamide. At a Bodipy-glibenclamide concentration of 10 nM, more than 90% of SURs should be occupied by Bodipy-glibenclamide (with regard to the K_D value given above). Tolbutamide has been shown to block K_{ATP} currents half-maximally at a concentration of 4 μM, and, in contrast to glibenclamide, the effects of

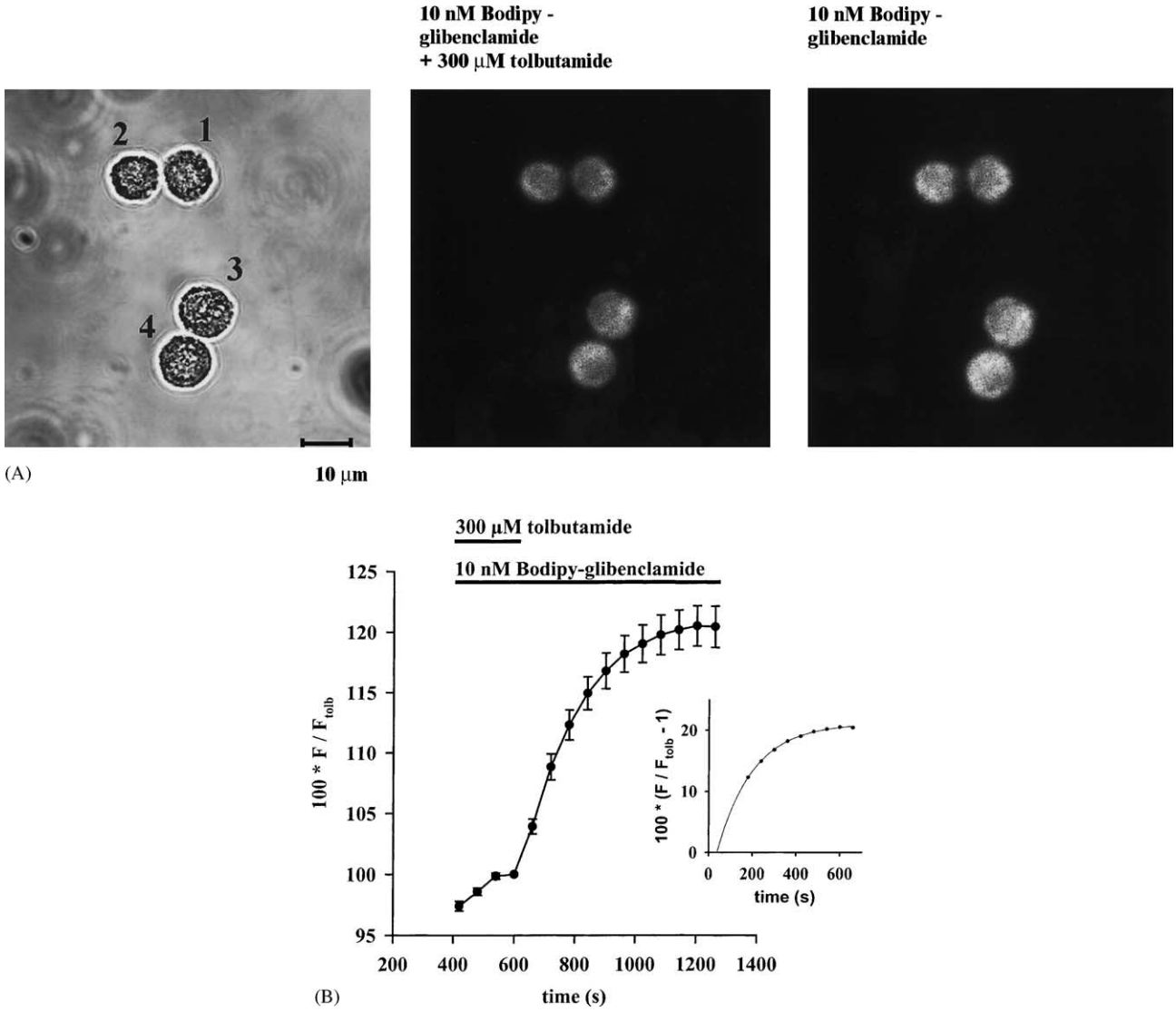

Fig. 3. Laser-scanning images of RINm5F cells. (A) Transmission image of RINm5F cells (left) and confocal images of the 10 nM Bodipy-glibenclamide-induced fluorescence of the cells in the presence (middle) and after 10 min of washout (right) of 300 μM tolbutamide. The intensity values of the optical sections with a diameter of 1 μm were the following for cells 1, 2, 3 and 4 in the presence and in the absence of tolbutamide, respectively: 86.1 and 102.6 for cell 1, 93.4 and 115.8 for cell 2, 100.3 and 122.4 for cell 3, 100.4 and 122.5 for cell 4. (B) Mean values ± SEM of Bodipy-glibenclamide-induced fluorescence intensity from 47 RINm5F cells pooled from 15 coverslips as a function of time. 10 nM Bodipy-glibenclamide was present throughout the experiments (lower horizontal bar), and the simultaneous presence of 300 μM tolbutamide is indicated by the upper horizontal bar. Ordinate: normalized Bodipy-glibenclamide-induced fluorescence intensity ($100(F/F_{tolb})$); abscissa: time. Points represent means and the vertical lines the SEM. Inset: the time-dependent increase of the Bodipy-glibenclamide-induced fluorescence intensity after washout of tolbutamide was fitted to Eq. (2). Due to the time required for the exchange of the bath solution (constant b in Eq. (2)), fitting of the exponential function started 3 min after beginning washout of tolbutamide. Ordinate: percent increase of the normalized fluorescence intensity ($100(F/F_{tolb} - 1)$); abscissa: time after beginning washout of tolbutamide. Points represent mean values calculated from the main figure.

tolbutamide on K_{ATP} channels are readily reversible within a few minutes washout [3]. In cells incubated in the presence of both 10 nM Bodipy-glibenclamide and 300 μM tolbutamide for 1–2 hr, it can be expected that most of the SURs are occupied by tolbutamide (the ratios of these concentrations to half-maximally inhibitory concentrations on K_{ATP} currents are 17 for Bodipy-glibenclamide and 75 for tolbutamide). The fluorescence intensity of RINm5F cells in the presence of both tolbutamide and Bodipy-glibenclamide (Fig. 3A, middle) might arise primarily from non-specific binding of Bodipy-glibenclamide, since most of the SUR1s are occupied by tolbutamide under these conditions. The increase of the fluorescence intensity after washout of tolbutamide (Fig. 3A, right) might be due to specific binding of Bodipy-glibenclamide to SUR1. In total 47 cells were studied on 15 coverslips. In all cells studied a significant increase of fluorescence intensity was observed after washout of tolbutamide. The time-dependent increase of the Bodipy-glibenclamide-induced fluorescence intensity after washout of tolbutamide was fitted to Eq. (2), and the values calculated for b, τ_{app} and C were 38.6 ± 5.8 s, 162 ± 7 s and 21.1 ± 0.1, respectively. Therefore, the calculated maximum increase of the Bodipy-glibenclamide-induced fluorescence intensity after washout of tolbutamide was 21.1% above the intensity in the simultaneous presence of tolbutamide. Note that after washout of tolbutamide, the Bodipy-glibenclamide-induced fluorescence intensity increased across the entire section of the cell (Fig. 3A, right).

In order to study whether the fluorescence label of Bodipy-glibenclamide contributes to the non-specific binding of Bodipy-glibenclamide observed in RINm5F cells (Fig. 3A, middle), the extent of binding of Bodipy-glibenclamide to albumin was determined using FCS. In these experiments, Bodipy-glibenclamide was tested at the same concentration (10 nM) as in laser-scanning confocal microscopy studies, and this concentration gave a sufficient signal-to-noise ratio. Depending on the laser intensity, the count rate per fluorescent molecule was up to 78 kHz for 10 nM Bodipy-glibenclamide (Fig. 4), and the number of Bodipy-glibenclamide molecules in the detection volume was between 1 and 2 at this concentration as determined by the autocorrelation function (3). The diffusion time constants were 32.5 ± 0.5 μs ($N = 20$) for rhodamine 6G (molecular weight = 478) and 37.7 ± 0.8 s ($N = 20$) for Bodipy-glibenclamide (molecular weight = 783), respectively. The diffusion time constant of Bodipy-glibenclamide was fixed in the autocorrelation functions where binding of Bodipy-glibenclamide to albumin was determined (Fig. 5). The diffusion time constant of albumin calculated according to the autocorrelation function (3) was 426.6 ± 22.6 μs ($N = 41$) indicating a molecular weight of about 1000 kDa, since for spherical molecules the diffusion time constant is proportional to the cubic root of the mass. This high molecular weight was probably due to an aggregation of albumin molecules. The extent of protein binding of 10 nM Bodipy-glibenclamide was $11.9 \pm 1.2\%$ at 10 μg/mL albumin ($N = 18$) and $34.9 \pm 1.4\%$ at 100 μg/mL albumin ($N = 15$); the latter value decreased to $13.6 \pm 2.0\%$ ($N = 8$) in the simultaneous presence of 1 μM non-labeled glibenclamide.

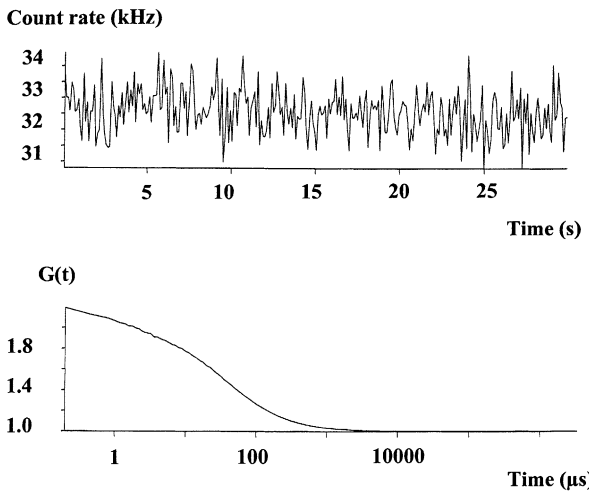

Fig. 4. Fluorescence intensity fluctuations (count rate; upper trace) and fluorescence autocorrelation function ($G(t)$; lower trace) of 10 nM Bodipy-glibenclamide. Bodipy-glibenclamide had a count rate of 32 kHz per molecule, and the diffusion time constant was 38 μs. A triplet state can be seen at short correlation times with a lifetime of 2 μs.

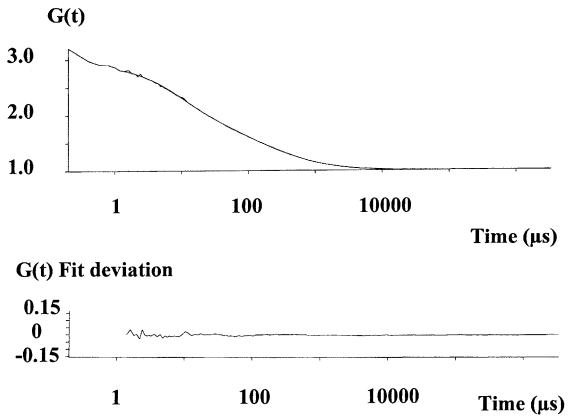

Fig. 5. Fluorescence autocorrelation function (upper trace) and residuals between simulated and recorded correlation function (lower trace) of a solution containing 10 nM Bodipy-glibenclamide and 100 μg/mL albumin. The fraction of Bodipy-glibenclamide bound to albumin was 37.4% and the diffusion time constant of albumin was 326 μs.

4. Discussion

The present study demonstrates that Bodipy-glibenclamide is a high-affinity blocker of pancreatic β-cell K_{ATP} currents and that specific binding of Bodipy-glibenclamide to SUR1 of a clonal insulinoma cell line can be demonstrated using laser-scanning confocal microscopy.

Both potency for block of K_{ATP} currents and binding affinity to SUR1 were only slightly lower for Bodipy-glibenclamide (IC_{50} value for channel block 0.6 nM, K_D value for binding 1.7 nM) when compared to glibenclamide (IC_{50} value 0.4 nM [3], K_D value 0.4 nM [4]). The potency of Bodipy-glibenclamide to inhibit the activity of K_{ATP} channels was about 3-fold higher than the binding affinity to microsomes from HIT-T15 cells. The discrepancy between binding and channel block has been suggested to result from the subunit architecture of K_{ATP} channels, and binding of sulfonylureas to any of the four sites per channel seems to be sufficient to induce closure of K_{ATP} channels [12].

Previously, Quesada *et al.* [7] visualized SURs in cultured β-cells from mouse pancreas using Bodipy-glibenclamide. The authors suggested that SUR1 was present in β-cells but not in glucagon-secreting α-cells since the Bodipy-glibenclamide-induced fluorescence intensity in β-cells was found to be 7-fold higher than in α-cells. Geng *et al.* [8] identified insulin secretory granules as the major site of K_{ATP} channels of the endocrine pancreas, since Bodipy-glibenclamide applied to freshly isolated mouse islet cells was localized to insulin secretory granules. However, it might be suggested that this binding in pancreatic β-cells was in part due to non-specific binding of Bodipy-glibenclamide due to its high lipophilicity. Glibenclamide has been shown to have a high lipophilicity, as determined by the partition of the compound between octanol and an aqueous phase (with an apparent partition coefficient of 94 [4]), and the lipophilicity of Bodipy-glibenclamide can be expected to be even higher (see the chemical structure in Fig. 1). When compared to the experiments published by Quesada *et al.* [7] and by Geng *et al.* [8], we first determined the potency for block of K_{ATP} currents by Bodipy-glibenclamide, used a lower Bodipy-glibenclamide concentration (10 nM vs. 40 nM) in order to reduce non-specific binding and determined specific binding of Bodipy-glibenclamide to SUR1 as the difference of the fluorescence intensity between the presence and absence of tolbutamide which rapidly dissociates from the SUR after washout [3]. Nevertheless, our results confirm those obtained by Quesada *et al.* [7] and by Geng *et al.* [8] demonstrating that SURs can be visualized in pancreatic β-cells using Bodipy-glibenclamide and laser-scanning confocal microscopy. However, as indicated by the effects of tolbutamide on the Bodipy-glibenclamide-induced fluorescence intensity from RINm5F cells in the present study, only about 17% of total Bodipy-glibenclamide-induced fluorescence intensity was due to specific binding to SUR1.

Specific binding of Bodipy-glibenclamide to SUR1 of RINm5F cells was detected across the entire section of the cell (Fig. 3A, right). Specific binding of Bodipy-glibenclamide to SUR1 localized to the plasma membrane was not discernible because it seemed to be masked by specific binding to SUR1 localized to intracellular structures and the plasma membrane labeling seems to be at the diffraction limit of the confocal microscope. Similarly, Geng *et al.* [8] observed little or no labeling of the β-cell plasma membrane by Bodipy-glibenclamide. After synthesis of plasma membrane proteins in the endoplasmic reticulum, they are forwarded to the Golgi complex, from which they are finally transported to the plasma membrane, and in immunofluorescence microscopy studies co-expressed SUR1 and Kir6.2 subunits have been shown to be present in these cellular components [13]. Similarly, in transfected COSm6 cells SUR1-GFP fluorescence localized to the endoplasmic reticulum, perinuclear space and plasma membrane. However, a strikingly different fluorescence pattern was observed when SUR1-GFP was cotransfected with Kir6.2, indicating a physical interaction: the perinuclear and reticular distribution was replaced by a diffuse fluorescence across the cell [14]. A specific binding site for sulfonylureas was also revealed by Bodipy-glibenclamide binding at the β-cell nuclear envelope [15]. Previously, the majority (>90%) of sulfonylurea binding sites have been shown to be localized to intracellular membranes with only minor levels (<10%) on plasma membranes of insulin-secreting cells, and sulfonylurea binding studies of insulinoma subcellular fractions showed that there was a high specific activity of receptors on secretory granule membranes [16]. This observation is in agreement with the observation of Geng *et al.* [8] that Bodipy-glibenclamide resulted in high-intensity signals of the insulin secretory granules relative to other cell structures. We suggest that the increase of the Bodipy-glibenclamide-induced fluorescence intensity after washout of tolbutamide in RINm5F cells might indicate specific binding to SUR1 localized to different intracellular structures (nuclear envelope, endoplasmic reticulum, Golgi compartment, insulin secretory granules) as well as binding to SUR1 localized to the plasma membrane. It is unknown whether insulin secretory granules are the major site of SUR1 of RINm5F cells, as previously shown for freshly isolated mouse islet cells [8].

Previously, a value of 17.2 s was determined for the half-life for the development of block of mouse pancreatic β-cell K_{ATP} currents by 100 nM glibenclamide [17]. Similar kinetics for drug–receptor interaction were observed in the present study using laser-scanning confocal microscopy, since specific binding of 10 nM Bodipy-glibenclamide to SUR1 of RINm5F cells increased with a half-life of 112 s (Fig. 3B, inset). From this value and a K_D value of 1.7 nM, an association rate constant of $5.3 \times 10^5\ s^{-1}\ M^{-1}$ and a dissociation rate constant of $9 \times 10^{-4}\ s^{-1}$ for binding of Bodipy-glibenclamide to SUR1 from RINm5F cells can be calculated [18].

The extent of binding of 10 nM glibenclamide to 2 mg/mL albumin, as determined previously by ultrafiltration, was about 84% at room temperature [4]. In the present FCS experiments, we could not test the extent of protein binding of Bodipy-glibenclamide at albumin concentrations higher than 100 μg/mL due to the autofluorescence of albumin. At 10 nM Bodipy-glibenclamide, protein binding

was concentration-dependent at albumin concentrations of 10 and 100 μg/mL. Overall, protein binding of Bodipy-glibenclamide seemed to be higher when compared to glibenclamide. This suggestion was supported by the observation that non-labeled glibenclamide (1 μM) could not completely inhibit the binding of Bodipy-glibenclamide (10 nM) to 100 μg/mL albumin, indicating that the fluorescence label contributes to the protein binding of Bodipy-glibenclamide. This observation might indicate that the fluorescence label also contributes to non-specific binding of Bodipy-glibenclamide to cellular components which might explain in part the fluorescence intensity of RINm5F cells observed in the presence of both Bodipy-glibenclamide and tolbutamide (Fig. 3A, middle).

In conclusion, Bodipy-glibenclamide is a high-affinity blocker of pancreatic β-cell K_{ATP} currents. The fluorescence label contributes to the protein binding of Bodipy-glibenclamide as determined by FCS. Using laser-scanning confocal microscopy specific binding of Bodipy-glibenclamide to SUR1 localized to intracellular structures could be observed, although non-specific binding markedly contributes to Bodipy-glibenclamide-induced fluorescence intensity of RINm5F cells.

Acknowledgments

We wish to thank Prof. Häberlein (University Bonn) for helpful comments and Dr. Kühne for performing some patch-clamp experiments.

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