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K^+ channel openers activate brain sulfonylurea-sensitive K^+ channels and block neurosecretion

(brain slices/$^{86}Rb^+$ efflux/γ-aminobutyric acid efflux/cromakalim)

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ABSTRACT **Vascular K^+ channel openers such as cromakalim, nicorandil, and pinacidil potently stimulate $^{86}Rb^+$ efflux from slices of substantia nigra. This $^{86}Rb^+$ efflux is blocked by antidiabetic sulfonylureas, which are known to be potent and specific blockers of ATP-regulated K^+ channels in pancreatic beta cells, cardiac cells, and smooth muscle cells. $K_{0.5}$, the half-maximal effect of the enantiomer (−)-cromakalim, is as low as 10 nM, whereas $K_{0.5}$ for nicorandil is 100 nM. These two compounds appear to have a much higher affinity for nerve cells than for smooth muscle cells. Openers of sulfonylurea-sensitive K^+ channels lead to inhibition of γ-aminobutyric acid release. There is an excellent relationship between potency to activate $^{86}Rb^+$ efflux and potency to inhibit neurotransmitter release.**

K^+ channel openers are a new class of molecules that have attracted general interest because of their potent antihypertensive activity *in vivo* and vasorelaxant activity *in vitro* (1). This class of compounds includes molecules of different structures, the best known of which are cromakalim, nicorandil, pinacidil, and minoxidil sulfate. They open K^+ channels and thereby hyperpolarize smooth muscle cells and decrease contraction. Both cromakalim and pinacidil (as well as its derivative P1075) have also been shown to be active in the heart cell; they shorten the action-potential duration and produce a negative inotropic effect (2–5). The effect of cromakalim in smooth muscle cells as well as in heart cells is inhibited by glibenclamide (2, 6, 7), an antidiabetic sulfonylurea that has been shown to be a specific blocker of ATP-regulated K^+ (K_{ATP}) channels (8). It is for this reason that it has been suggested that K^+ channel openers actually act on K_{ATP} channels (2, 6, 7, 9).

The purpose of this paper is to gain more information concerning the mechanism of action of these K^+ channel openers in the nervous system, which is known to contain both K_{ATP} channels (10) and sulfonylurea receptors (11–13). The brain area that has been selected for this work is the substantia nigra (SN), a region that has been found to have the highest density of receptors for sulfonylureas (14). Cromakalim, nicorandil, and pinacidil do open K^+ channels in this important area of the central nervous system, and these channels have an important role in the release of the inhibitory transmitter γ-aminobutyric acid (GABA) from SN terminals.

MATERIALS AND METHODS

Materials. 4-Amino-*n*-[2,3-^{3}H]butyric acid ([^{3}H]GABA; 90 Ci/mmol; 1 Ci = 37 GBq) and ^{86}RbCl (1–8 Ci/mg) were from Amersham. Aminooxyacetic acid was from Sigma. Glibenclamide, tolbutamide, and LH32, were from Hoechst–Roussel; gliquidone was from Boehringer Ingelheim; glipizide was from Pfizer; cromakalim and (−)-cromakalim were from Beecham Pharmaceuticals; pinacidil and P1075 were from Leo Pharmaceuticals (Helsingborg, Sweden); nicorandil was from Rhône-Poulenc; and minoxidil sulfate was from Upjohn.

Preparation of Slices. Wistar rats of 200 g were killed by decapitation, and their brains were rapidly removed. SN slices (0.7 mm) were dissected with a McIlwain chopper and rapidly incubated at 37°C in medium A [11 mM Hepes·NaOH (pH 7.4) containing 120 mM NaCl, 3.5 mM KCl, 1.0 mM $MgSO_4$, 16 mM $NaHCO_3$, 1.2 mM $CaCl_2$, and 10 mM D-glucose] previously gassed with 95% O_2/5% CO_2. Slices were gassed for 90 min before the beginning of the experiments to permit a full recovery. The aeration was continuous during the whole procedure.

Release of [^{3}H]GABA. Slices were preloaded for 20 min with 1 μM [^{3}H]GABA (2 μCi/ml) in 1 ml of medium A in the presence of 50 μM aminooxyacetic acid to inhibit GABA transaminase. A 45-min washing with medium A was done before beginning the release experiments. Then one slice was transferred per well of a multiwell box (1 ml of oxygenated medium A per well) as described (15). Release experiments were then carried out during consecutive intervals of 5 min for 40–60 min. Eight independent experiments were performed at the same time.

Efflux of $^{86}Rb^+$. Slices were preloaded for 30 min with 5–10 μCi of $^{86}Rb^+$ per ml in 2 ml of medium A. A 30-min washing with medium A was done before starting the $^{86}Rb^+$ efflux. Experiments were then performed as for [^{3}H]GABA release.

Data Analysis. Fractional rates of release were calculated as [^{3}H]GABA or $^{86}Rb^+$ released during each 5-min interval and expressed as the percentage of the [^{3}H]GABA or $^{86}Rb^+$ content in the tissue at the beginning of the respective intervals (15). Individual experiments were performed with several wells (2–3), and experiments were repeated *n* times. The results were calculated as the mean of all experiments ± SEM. $K_{0.5}$ values (half-maximal effects) were determined by least-squares fit of the data to a sigmoidal function as described (16).

For dose–response curves, basal rates of $^{86}Rb^+$ and of [^{3}H]GABA efflux were subtracted at the level of the top peak from activated rates as described (15).

RESULTS

The activity of cromakalim-sensitive K^+ channels in smooth muscle cells has previously been successfully studied with $^{86}Rb^+$-efflux experiments (9, 17). $^{86}Rb^+$-efflux techniques have also been used with success to characterize the phar-

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Abbreviations: K_{ATP}, ATP-regulated potassium channel; SN, substantia nigra; GABA, γ-aminobutyric acid.

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macological properties and the mechanism of regulation of K_{ATP} channels in insulinoma cells (18). Typical kinetics of $^{86}Rb^+$ efflux from SN slices are shown in Fig. 1*A*. (−)-Cromakalim, the active enantiomeric form of cromakalim, creates a transient component of $^{86}Rb^+$ efflux that is maximum (1.7 times activation) in the first 5-min period of incubation with 100 nM (−)-cromakalim. This activation of $^{86}Rb^+$ efflux by (−)-cromakalim is inhibited by 100 nM gliquidone, one of the most powerful sulfonylureas (18). Among other K^+ channel openers tested, only minoxidil sulfate had no detectable effect up to 100 μM.

Concentration dependences of activations of $^{86}Rb^+$ efflux by (−)-cromakalim and other K^+ channel openers are shown in Fig. 1*B*. Half-maximal concentrations for activation vary between 10 nM and 500 nM. The rank order of potency of the different K^+ channel openers is (−)-cromakalim > nicorandil > pinacidil and P1075 > cromakalim.

Fig. 2 presents an analysis of the efficacy of gliquidone and a series of other sulfonylureas for inhibiting the (−)-cromakalim-activated $^{86}Rb^+$ efflux. Half-maximal inhibition values of the different sulfonylureas vary between 0.4 nM and 460 nM [gliquidone ($K_{0.5}$ = 0.4 ± 0.1 nM) > glipizide ($K_{0.5}$ = 5 ± 1 nM) > LH32 ($K_{0.5}$ = 200 ± 10 nM) > glibenclamide ($K_{0.5}$ = 460 ± 20 nM)].

We have recently demonstrated that both glucose and sulfonylureas activate neurotransmitter release in SN through an inhibition of K_{ATP} channels (19).

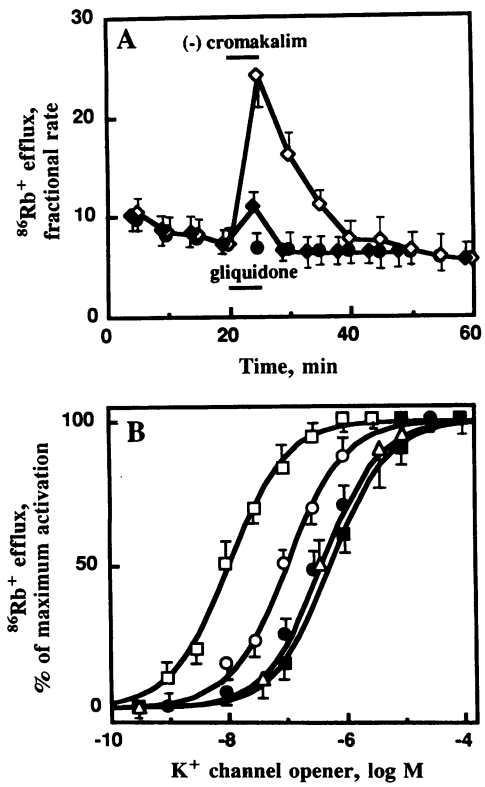

FIG. 1. Kinetics of $^{86}Rb^+$ efflux evoked by (−)-cromakalim and activation by different K^+ channel openers in SN slices. (*A*) Kinetics of $^{86}Rb^+$ efflux without (●) or with (◇) 100 nM (−)-cromakalim and in the absence (◇) or presence (◆) of 100 nM gliquidone. Horizontal bars represent the period of application of the effectors (n = 5). In experiments in which both (−)-cromakalim and gliquidone are used, the two drugs are added at the same time. (*B*) Activation of $^{86}Rb^+$ efflux was measured in the presence of increasing concentrations of (−)-cromakalim (□) ($K_{0.5}$ = 10 ± 2 nM), nicorandil (○) ($K_{0.5}$ = 100 ± 10 nM), pinacidil (●) and P1075 (△) ($K_{0.5}$ = 400 ± 15 nM), and cromakalim (■) ($K_{0.5}$ = 500 ± 20 nM). The maximal activation was the same for all compounds tested. Time of activation of $^{86}Rb^+$ efflux was 5 min (n = 4).

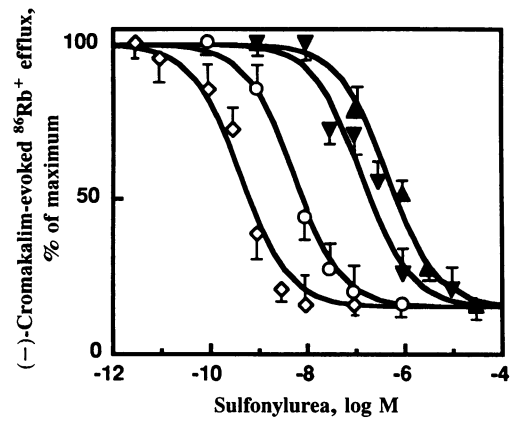

FIG. 2. Inhibition by different sulfonylureas of $^{86}Rb^+$ efflux activated by 100 nM (−)-cromakalim. $^{86}Rb^+$ efflux was inhibited by increasing concentrations of gliquidone (◇) ($K_{0.5}$ = 0.4 ± 0.1 nM), glipizide (○) ($K_{0.5}$ = 5 ± 1 nM), LH32 (▼) ($K_{0.5}$ = 200 ± 10 nM), and glibenclamide (▲) ($K_{0.5}$ = 460 ± 20 nM). Time of activation of $^{86}Rb^+$ efflux was 5 min (n = 4).

Thus, at this point it appeared important to study the potential effect of K^+ channel openers on the evoked release of GABA, which is the main neurotransmitter in SN. Release of [^{3}H]GABA from preloaded slices could be evoked either by 15 mM K^+, by 20 mM glucose, or by 100 nM gliquidone as shown in Fig. 3*A Inset*. All of these different types of activations of [^{3}H]GABA release were similarly inhibited by increasing concentrations of (−)-cromakalim with a $K_{0.5}$ value of 10 ± 2 nM.

Fig. 3*B* presents an analysis of the efficacy of the series of K^+ channel openers in inhibiting [^{3}H]GABA release evoked by 100 nM gliquidone. $K_{0.5}$ values for the inhibition by the different openers vary between 10 nM and 500 nM. The rank order of potency of the openers was: (−)-cromakalim > nicorandil > cromakalim > pinacidil > P1075. Minoxidil sulfate, which was inactive in $^{86}Rb^+$ efflux experiments, was also inactive in inhibiting [^{3}H]GABA release.

Fig. 4 shows that an excellent linear relation in the structure–function relationship is observed between the affinities of the different K^+ channel openers for inhibiting the gliquidone-activated [^{3}H]GABA release and the affinities of the different openers for stimulating $^{86}Rb^+$ efflux from SN slices.

DISCUSSION

The description of the mechanism of action of cromakalim (1, 20) has triggered a considerable interest for drugs that open K^+ channels in smooth muscle cells (1, 20). There are already a plethora of therapeutic indications in which K^+ channel openers are expected to have a beneficial effect, such as hypertension, asthma, cardiac failure, angina pectoris, irritable bladder, and probably other smooth muscle pathologies.

It is only recently (21–23) that an action of these openers on the central nervous system has been recognized. Cromakalim has been shown to reduce excitability in CA_3 hippocampal cells (21) and to display antiepileptic properties in a model of drug-induced epilepsia (22, 23). Therefore, it appeared to us that it was important to try to establish the molecular basis of the action of these compounds in brain cells. The choice of SN to study the mechanism of action of cromakalim, pinacidil, nicorandil, and their analogs was linked to the fact that, both in smooth muscle and in cardiac muscle (2, 6, 20, 24), the target of these drugs seems to be the sulfonylurea-sensitive K_{ATP} channel. It is in SN that one finds the highest amount of sulfonylurea receptors in the brain (14).

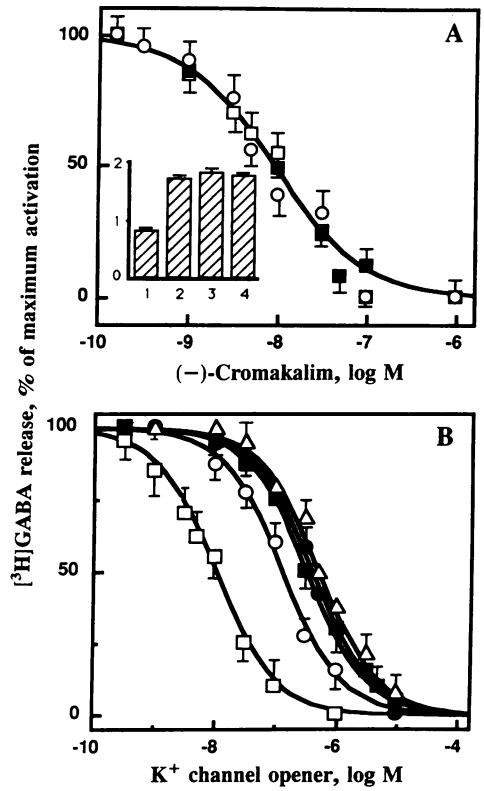

FIG. 3. Inhibition of [^{3}H]GABA-evoked release from SN slices by increasing concentrations of (−)-cromakalim. Release was evoked either by 15 mM K^+ (○), by 20 mM glucose (□), or by 100 nM gliquidone (■). The basal release of [^{3}H]GABA in 3.5 mM K^+ medium was not affected by 100 nM (−)-cromakalim. (*Inset*) Activation by different effectors of [^{3}H]GABA release. Columns: 1, basal release in 3.5 mM K^+-containing medium A; 2, 15 mM K^+; 3, 20 mM glucose; 4, 100 nM gliquidone. Time of [^{3}H]GABA-evoked release was 5 min. Ordinate shows [^{3}H]GABA release in fractional rate (n = 4). (*B*) Inhibition by different K^+ channel openers of [^{3}H]GABA release evoked by 100 nM gliquidone. This release was inhibited by increasing concentrations of (−)-cromakalim (□) ($K_{0.5}$ = 10 ± 2 nM), nicorandil (○) ($K_{0.5}$ = 120 ± 5 nM), cromakalim (■) ($K_{0.5}$ = 300 ± 15 nM), pinacidil (●) ($K_{0.5}$ = 400 ± 20 nM), and P1075 (△) ($K_{0.5}$ = 500 ± 20 nM) (n = 4).

All potential brain K^+ channel openers tested in this work produced the effects expected for drugs that would open K^+ channels. They triggered $^{86}Rb^+$ efflux from SN as they do in smooth muscle (1, 9, 20), and this efflux was inhibited by sulfonylureas.

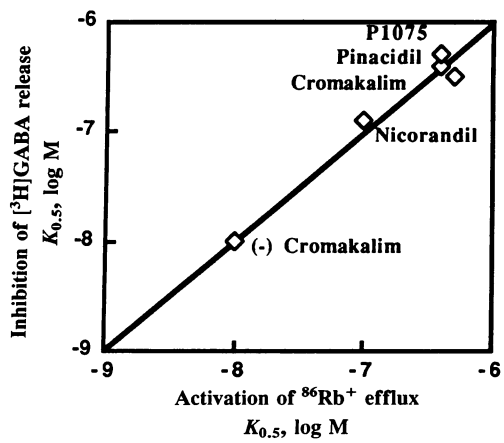

FIG. 4. Structure–function relationships for the effect of K^+ channel openers on inhibition of [^{3}H]GABA release and activation of $^{86}Rb^+$ efflux. $K_{0.5}$ values of different K^+ channel openers for inhibition of gliquidone-evoked [^{3}H]GABA release were plotted versus $K_{0.5}$ values of the same compounds for activation of $^{86}Rb^+$ efflux (slope of line = 1.01 ± 0.03).

The most obvious conclusion from this work is that cromakalim, nicorandil, pinacidil, and parent compounds open sulfonylurea-sensitive K^+ channels in SN. Minoxidil sulfate, which is active in smooth muscle at concentrations in the 0.1–1 μM range (25, 26) and used therapeutically (25), is inactive on SN at concentrations as high as 100 μM.

Targets of K^+ channel openers in SN are not identical to those they have in smooth muscle cells: (*i*) the apparent affinity ($K_{0.5}$) of (−)-cromakalim and nicorandil for SN is 50–1000 times higher than in the smooth muscle cell [(−)-cromakalim: $K_{0.5}$(SN) = 10 nM versus $K_{0.5}$(portal vein) = 500 nM (20), and nicorandil: $K_{0.5}$(SN) = 100 nM versus $K_{0.5}$(portal vein) = 100 μM (20)]; (*ii*) the order of efficacy is also different—e.g., pinacidil is much more potent than nicorandil in its action on smooth muscle cells [$K_{0.5}$(pinacidil) = 5 μM > $K_{0.5}$(nicorandil) = 100 μM (20)] but is much less potent in SN (Fig. 1); and (*iii*) pinacidil and its analog P1075 are nearly equipotent on K^+ channels in SN (Fig. 1), whereas P1075 is much more potent on portal vein [$K_{0.5}$(pinacidil) = 5 μM, $K_{0.5}$(P1075) = 300 nM) (20)] and also in heart cells [$K_{0.5}$(pinacidil) = 2 μM, $K_{0.5}$(P1075) = 37 nM) (4)].

K^+ channel openers have an interesting inhibitory effect on GABA release from SN terminals. The good linear relation between $^{86}Rb^+$ efflux and [^{3}H]GABA release data (Fig. 4) suggests that the inhibition of the neurotransmitter release, like the inhibition of contraction in smooth muscle cells (1, 20), is linked to the K^+ channel-opening capacity of these compounds. The most probable mechanism of action is: (*i*) opening of K^+ channels, (*ii*) hyperpolarization of previously depolarized terminals (in SN pars reticulata) to a new membrane potential value far from the threshold of activation of Ca^{2+} channels that provide Ca^{2+} entry for GABA secretion, and (*iii*) inhibition of Ca^{2+} entry and thereby of GABA secretion. A similar mechanism has been proposed to explain the inhibitory effect of hormones such as galanin and somatostatin in insulin secretion from pancreatic beta cells (27–29).

All ionic channel openers have become important tools in neurobiological research. This is particularly the case for openers of voltage-sensitive Na^+ channels, such as veratridine and batrachotoxin (30, 31), or for openers of voltage-sensitive L-type Ca^{2+} channels, such as Bay K 8644 (32–35). Moreover, while a lot of procedures are available to depolarize membranes [K^+ depolarization, veratridine or batrachotoxin treatment, ouabain inhibition of (Na^+,K^+)-ATPase, etc.], creating a hyperpolarization is always more difficult. Therefore, compounds such as (−)-cromakalim or nicorandil will undoubtedly become tools of a very general use in neurobiology.

Another potential application of the results presented in this work concerns the identification of the receptors of K^+ channel openers using radiolabeled ligands. Such an approach has not been successful until now in smooth muscle cells probably because of the relatively modest affinity of the drug for their binding component. The situation is different in SN in which affinities in the 10 nM range for (−)-cromakalim should permit a characterization of the receptor site. A receptor characterization might even be possible with labeled nicorandil. A biochemical identification of the binding site of at least one of these compounds would be essential to know whether all of the different compounds presented in Fig. 1 bind to the same receptor site, or else, if there are, as for Ca^{2+} channel effectors (32), several types of receptor sites for the different families of compounds.

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