Involvement of Pertussis Toxin-sensitive G-proteins in the Hormonal Inhibition of Dihydropyridine-sensitive Ca\textsuperscript{2+} Currents in an Insulin-secreting Cell Line (RINm5F)*

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Adrenaline inhibits insulin secretion via pertussis toxin-sensitive mechanisms. Since voltage-dependent Ca\textsuperscript{2+} currents play a key role in insulin secretion, we examined whether adrenaline modulates voltage-dependent Ca\textsuperscript{2+} currents of the rat insulinoma cell line, RINm5F. In the whole-cell configuration of the patch-clamp technique, dihydropyridine- but not \(\omega\)-conotoxin-sensitive Ca\textsuperscript{2+} currents were identified. Adrenaline via \(\alpha_2\)-adrenoceptors inhibited the Ca\textsuperscript{2+} currents by about 50\%. Somatostatin which also inhibits insulin secretion was less efficient (inhibition by 20\%). The hormonal inhibition of Ca\textsuperscript{2+} currents was not affected by intracellularly applied cAMP but blocked by the intracellularly applied GDP analog guanosine 5']-O-(2-thiodiphosphate) and by pretreatment of cells with pertussis toxin. In contrast to adrenaline and somatostatin, galanin, another inhibitor of insulin secretion, reduced Ca\textsuperscript{2+} currents by about 40\% in a pertussis toxin-insensitive manner. Immunoblot experiments performed with antibodies generated against synthetic Guilt Gi2, Go2, and another Go subtype, most likely representing Go1. In membranes of control but not of pertussis toxin-treated cells, adrenaline via \(\alpha_2\)-adrenoceptors stimulated incorporation of the photoreactive GTP analog [\(\alpha\textsuperscript{32P}\)]GTP azidoanilide into pertussis toxin substrates comigrating with the \(\alpha\)-subunits of Gi2, Go2, and not further identified Go subtype. The present findings indicate that activated \(\alpha_2\)-adrenoceptors of RINm5F cells interact with multiple G-proteins, \textit{i.e.} two forms of Gi, and with Go. These G-proteins are likely to be involved in the adrenaline-induced inhibition of dihydropyridine-sensitive Ca\textsuperscript{2+} currents and in other signal transduction pathways contributing to the adrenaline-induced inhibition of insulin secretion.

In pancreatic B-cells, metabolism of glucose leads to a closure of ATP-dependent K\textsuperscript{+} channels and subsequent opening of voltage-dependent Ca\textsuperscript{2+} channels and increased Ca\textsuperscript{2+} influx (1). In addition, glucose metabolism increases Ca\textsuperscript{2+} influx by directly modulating the activity of voltage-dependent Ca\textsuperscript{2+} channels (2, 3). The influx of Ca\textsuperscript{2+} through voltage-dependent Ca\textsuperscript{2+} channels and associated changes in intracellular Ca\textsuperscript{2+} appear to be causally related to insulin secretion (4). Similar to secretory processes in other cell types (5), insulin release from permeabilized cells can also be observed at extremely low Ca\textsuperscript{2+} concentrations (6). Thus Ca\textsuperscript{2+}-dependent and \textit{independent} mechanisms of insulin secretion may exist.

Adrenaline (via \(\alpha_2\)-adrenoceptors), somatostatin, and galanin inhibit insulin secretion (1). The inhibitory effects of these hormones are mediated by G-proteins\textsuperscript{1} (for recent reviews see Refs. 7, 8) which are substrates for pertussis toxin (9). In fact, pertussis toxin-induced hypoglycemia, due to an increase in insulin secretion, led to the original denomination of the toxin as "islet-activating protein" by Ui and coworkers (10). The precise molecular mechanisms by which hormones inhibit insulin secretion are not known. Somatostatin and galanin but not adrenaline have been shown to stimulate ATP-dependent K\textsuperscript{+} channels via pertussis toxin-sensitive mechanisms (11, 12), thereby causing hyperpolarization of the plasma membrane and closure of voltage-dependent Ca\textsuperscript{2+} channels. In permeabilized rat insulinoma cells (RINm5F), adrenaline and galanin have been shown to inhibit insulin secretion in a pertussis toxin-sensitive manner, suggesting a direct effect of pertussis toxin-sensitive G-proteins on the secretory process (13, 14). Analogous observations were recently made with somatostatin in SV40-transformed hamster pancreatic B-cells (HIT) (15).

In neuronal and pituitary cells, secretion-inhibiting hormones and neurotransmitters inhibit voltage-dependent Ca\textsuperscript{2+} channels via pertussis toxin-sensitive G-proteins; this inhibition is direct, \textit{i.e.} not due to stimulation of hyperpolarizing currents (16, 17). In addition, opening of neuronal N-type

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\1 The abbreviations used are: G-proteins, a family of heterotrimeric guanine nucleotide-binding regulatory proteins; Gi, a cholea toxin-sensitive G-protein involved in the receptor-mediated stimulation of adenylylcyclase; pertussis toxin, an exotoxin of Bordetella pertussis; G, (subtypes Gi1, Gi2, Gi3) and G, (subtypes Go1, Go2, Go3) homologous, pertussis toxin-sensitive G-proteins involved in receptor-mediated modulations of enzymes and ion channels; transducin, retinal G-protein involved in the light-mediated stimulation of cGMP-phosphodiesterase; Go, a cholea and pertussis toxin-insensitive G-protein of unknown function; SDS, sodium dodecyl sulfate.
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For pharmacological characterization, Ca\(^{2+}\) currents were evoked by depolarizing pulses from -80 to 0 mV. The dihydropyridine, isradipine (1 \(\mu\)M), caused an inhibition of Ca\(^{2+}\) currents by 80 ± 12% (mean ± S.D., \(n = 7\)) (Fig. 2A): removal of the compound from the bath led to an essentially complete recovery of currents. In contrast, the N-type channel blocker \(\omega\)-conotoxin (22-24) employed at a high concentration (10 \(\mu\)M) was virtually inactive (Fig. 2B). The current decrease observed during superfusion of cells with the toxin (9 ± 8%; mean ± S.D., \(n = 5\)) may rather reflect the rapid rundown of currents in these cells than a specific effect of the toxin. The data show that the by far major portion of Ca\(^{2+}\) currents in RINm5F cells represents currents through dihydropyridine-sensitive channels.

Extracellular application of adrenaline (10 \(\mu\)M) in the presence of propranolol (1 \(\mu\)M) led to an inhibition of Ca\(^{2+}\) currents by 48 ± 7% (mean ± S.D., \(n = 14\)) (Fig. 2, Ca and Cb). The maximal inhibition was observed within 20 s. After removal of adrenaline, currents increased immediately and within about 60 s reached a maximal amplitude close to that of control currents. The reversible inhibition of Ca\(^{2+}\) currents by the hormone was observed in all cells tested. Adrenaline also appeared to reduce the initial rate of current inactivation. The adrenaline-induced effect was not affected by pretreatment of cells with \(\omega\)-conotoxin (five experiments; Fig. 2, Da and Db). This finding indicates that, in contrast to neuronal cells (18), the agonist-sensitive Ca\(^{2+}\) currents of RINm5F cells do not represent currents through N-type channels but at least in part through dihydropyridine-sensitive channels.

The involvement of G-proteins in the inhibitory Ca\(^{2+}\) cur-

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Ca\(^{2+}\) currents through voltage-dependent Ca\(^{2+}\) channels were evoked by 200-ms pulses from holding potentials of -80 or -40 mV (Fig. 1, A and B). Under both conditions, inward currents with a slow time course of inactivation were observed. At a holding potential of -80 mV, a minor rapidly inactivating current component was observed in most cells. At a test potential of 0 mV, this rapidly inactivating current component contributed less than 15% to the maximal peak current. In contrast, other endocrine cells including pituitary GH3 cells and adrenocortical Y1 cells possess large low threshold, fast inactivating currents (20, 21). Applying a test potential of 0 mV, the maximal peak current amplitude amounted to 247 ± 106 pA at a holding potential of -80 mV (mean value ± S.D., \(n = 21\)) and to 205 ± 64 pA at a holding potential of -40 mV (mean value ± S.D., \(n = 7\)). Within the 200-ms test pulse, the currents elicited by depolarizing pulses from either holding potential inactivated by about 40%. The current-voltage curves obtained from the two holding potentials were similar (Fig. 1C). Maximal peak current amplitudes were observed at a test potential of about -10 mV; the apparent threshold and reversal potentials amounted to about -40 and +30 mV, respectively. “Rundown” of currents started immediately after disruption of the membrane patch; within 10 min the maximal peak current amplitude was reduced by 50% irrespective of the holding potential.

*Portions of this paper (including “Experimental Procedures” and Figs. 7-11) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.*
rent modulation is demonstrated by the experiments shown in Fig. 2, E and F. If the GDP analog, guanosine 5'-O-(2-thiodiphosphate) (100 µM), was infused into cells via the patch pipette for 5 min, an inhibitory effect of adrenaline was no longer observed (inhibition by 4 ± 3%; mean ± S.D., n = 4).

Similar, pretreatment of cells with pertussis toxin abolished the Ca" current inhibition induced by adrenaline (inhibition by 4 ± 4%; mean ± S.D., n = 5). The data show that a pertussis toxin-sensitive G-protein is involved in the inhibitory modulation of Ca" currents.

Other receptor agonists known to inhibit insulin secretion (see introduction) also attenuated Ca" currents in RINm5F cells. Somatostatin (1 µM) was less efficient than adrenaline; the peptide reversibly reduced Ca" currents by 19.8 ± 2.2% (mean ± S.D., n = 5). Like the adrenaline-induced Ca" current inhibition, the effect of somatostatin was abolished by pretreatment of cells with pertussis toxin (Ca" current inhibition by 0.8 ± 0.8%; mean ± S.D., n = 4). In contrast, the reversible Ca" current inhibition induced by galanin (1 µM) was not affected by pertussis toxin. The peptide inhibited Ca" currents in control and pertussis toxin-treated cells by 39.1 ± 7.5% (mean ± S.D., n = 9) and 35.7 ± 8.7% (mean ± S.D., n = 5), respectively. Since galanin stimulates ATP-dependent K" channels in a pertussis toxin-sensitive manner (indicating a coupling of galanin receptors to pertussis toxin sensitive G-proteins), its effect on Ca" currents in RINm5F cells may not require receptor activation. Alternatively, galanin receptors may additionally couple to G-proteins which are not substrates for pertussis toxin. In this context, it is of interest that adrenaline and somatostatin but not galanin inhibit insulin secretion in a GTP-dependent manner in permeabilized RINm5F and HIT cells (15). The present data indicate that somatostatin and galanin inhibit insulin secretion not only by stimulation of ATP-regulated K" channels (11, 12), but also by inhibition of voltage-dependent Ca" channels.

The inhibitory effect of adrenaline (10 µM; in the presence of 1 µM propranolol) was pharmacologically characterized. It was abolished by the α2-adrenoceptor antagonist, yohimbine (100 µM; three experiments), but not affected by the α1-adrenoceptor antagonist, prazosin (100 µM; three experiments) (data not shown). The α2-adrenoceptor agonist, clonidine, mimicked the inhibition of Ca" currents by adrenaline. Clonidine (10 µM) inhibited Ca" currents by 48 ± 9% (mean ± S.D., n = 8). The data indicate that the inhibitory Ca" current modulation induced by adrenaline requires activation of α2-adrenoceptors.

Since adrenaline via α2-adrenoceptors and pertussis toxin-sensitive G-proteins inhibits adenylcyclase, we examined the effect of CAMP on the inhibitory Ca" current modulation. Intracellular application of CAMP (100 µM in the pipette solution) for 10 min did not significantly affect the rapid rundown of currents nor the response of cells to adrenaline applied 5 min after disruption of the membrane patch (current inhibition by 41.5 ± 6.2%, mean ± S.D., n = 4). Thus the inhibitory effect of adrenaline on Ca" currents does not involve a CAMP-dependent intermediate step.

The inhibitory Ca" current modulation involves pertussis toxin-sensitive G-proteins. Therefore we examined the effects of adrenaline and clonidine on high-affinity GTPase in membranes of RINm5F cells (Fig. 3; see also "Experimental Procedures," Figs. 10 and 11). Adrenaline (30 µM, in the presence of 10 µM propranolol) and clonidine (30 µM) stimulated high-affinity GTPase by about 50–60% and 20–30%, respectively. Like in other systems (25), the basal GTPase activity was reduced in membranes of pertussis toxin-treated cells, and neither adrenaline nor clonidine caused an increase in GTPase activity. The data show that activated α2-adrenoceptors promote to a measurable extent the nucleotide exchange of pertussis toxin-sensitive G-proteins in membranes of RINm5F cells.

For identification of pertussis toxin-sensitive G-proteins in RINm5F cells, membranes were probed with the photoreactive GTP analog, [α-32P]GTP azidoanilide, and with antibodies directed against G-protein α-subunits (Fig. 4, see also "Experimental Procedures," Figs. 7–9). In the experiments shown in Fig. 4, A and B, membranes of RINm5F cells were incubated with [α-32P]GTP azidoanilide, and proteins were separated by SDS-polyacrylamide gel electrophoresis (see above) and blotted onto nitrocellulose filters. Shown is the autoradiogram of the filters. Molecular masses of marker proteins are indicated by arrowheads.
and then incubated with antibodies; filter-bound antibodies were visualized by a color reaction.

In photolabeled membranes, an \( \alpha_{\text{common}} \)-peptide antibody, which recognizes the \( \alpha \)-subunits of \( \text{Gi}_1, \text{Gi}_2, \text{Go}_2, \text{Ga}_1, \text{Go}_2, \text{Gi}, \) and transducin, reacted with proteins of 39–43 kDa (see Fig. 4A). An \( \alpha_{\text{common}} \)-peptide antibody, which recognizes \( \text{Gi}, \) and \( \text{Go}_2 \) \( \alpha \)-subunits, detected a poorly resolved 42/41-kDa doublet and a broad 39-kDa band. A recently developed \( \alpha_{\text{a2}} \) peptide antibody which is specific for the \( \text{Ga}_1 \) \( \alpha \)-subunit (26) reacted with a protein comigrating with the 39-kDa protein recognized by the \( \alpha_{\text{common}} \) peptide and \( \alpha_{\text{common}} \) Peptide antibodies. The \( \alpha_{\text{common}} \) peptide antibody, which recognizes the various \( \text{G} \) \( \alpha \)-subunits, reacted with a 43/42-kDa doublet and a broad 40-kDa band. As is shown in Fig. 9 ("Experimental Procedures"), the 43/42-kDa doublet is also recognized by an antibody specific for the \( \text{Gi}_1 \) \( \alpha \)-subunit, and the 40-kDa band is recognized by an antibody specific for the \( \text{Go}_2 \) \( \alpha \)-subunit. The latter most likely corresponds to the \( \text{Gi}_1 \) \( \alpha \)-subunit since (i) only two forms of the \( \text{G} \) \( \alpha \)-subunits are known on the protein level (\( \text{Ga}_1 \) and \( \text{Ga}_2 \) \( \alpha \)-subunits; 27), and (ii) Hsu and coworkers (28) detected mRNA encoding the \( \text{Go}_1 \) \( \alpha \)-subunit in RNA extract of \( \text{RINm5F} \) cells. The appearance of the slower migrating \( \text{Gi}, \) \( \alpha \)-subunit and the \( \text{Gi}_1 \) \( \alpha \)-subunit as doublets and that of the \( \text{Go}_2 \) and the \( \text{Ga}_2 \) \( \alpha \)-subunit as broad bands may indicate that these subunits exist in different forms which differ in their posttranslational modifications. \( \text{Gn}_2 \) \( \alpha \)-subunits were not detected in membranes of \( \text{RINm5F} \) cells (see Fig. 9, "Experimental Procedures").

The apparent molecular masses of some of the \( \text{G} \)-protein \( \alpha \)-subunits (see Fig. 4) are higher than those reported by other groups (7, 8). This abnormal behavior is entirely due to urea, which apparently unfolds \( \text{G} \)-protein \( \alpha \)-subunits by varying degrees, thereby differentially reducing their mobility in SDS-gels. Urea employed at concentrations \( \geq 6 \) M was crucial to achieve resolution of the \( \text{Gn}_2 \) \( \alpha \)-subunit and the slower migrating \( \text{Gi}, \) \( \alpha \)-subunit (compare Fig. 4 with Fig. 8 in "Experimental Procedures"). A decrease in the urea concentration from 6 to 4 M led to M, values which were close to the widely reported values (7, 8).

The fuzzy appearance of photolabeled proteins of 39–43 kDa (see Fig. 4) arises from the transfer of proteins from gels containing high concentrations of urea onto nitrocellulose filters since photolabeled proteins were well focused if SDS-gels were directly autoradiographed (compare Fig. 4 with Figs. 5 and 6). The mobility of the major photolabeled protein was indistinguishable from that of the 42/41-kDa doublet recognized by the \( \alpha_{\text{common}} \) peptide and \( \alpha_{\text{common}} \) peptide antibodies (see Fig. 4A). A photolabeled protein of 40 kDa comigrated with the protein recognized by the \( \alpha_{\text{common}} \) and \( \alpha_{\text{a2}} \) peptide antibodies. A photolabeled protein of 39 kDa migrated with the protein recognized by the \( \alpha_{\text{a1}} \) and \( \alpha_{\text{a2}} \) peptide antibodies. The upper minor photolabeled protein comigrated with the 43/42-kDa doublet recognized by the \( \alpha_{\text{common}} \) and \( \alpha_{\text{a1}} \) peptide antibodies. The data indicate that the photolabeled proteins correspond to the \( \alpha \)-subunits of \( \text{Gi}_1, \text{Gi}_2, \text{Ga}_1, \) and \( \text{Go}_2 \). Indistinguishable mobilities of photolabeled proteins and immunologically detected \( \text{G} \)-protein \( \alpha \)-subunits were also observed with SDS-gels containing 4 M urea; in addition, blotted photolabeled \( \text{G} \)-protein \( \alpha \)-subunits appeared as sharp bands (see Fig. 8, "Experimental Procedures").

To demonstrate that the photolabeled proteins were substrates for pertussis toxin, we took advantage of the observation that modification of \( \text{G} \)-protein \( \alpha \)-subunits by pertussis toxin decreases their mobility in urea-containing SDS-gels (29). In membranes of pertussis toxin-treated cells, the mobility of photolabeled and immunoreactive proteins was reduced by the same extent (see Fig. 4B; see also Fig. 8, "Experimental Procedures"). Pertussis toxin differently reduced the mobility of \( \text{G} \)-protein \( \alpha \)-subunits, resulting in comigration of the \( \text{Ga}_1 \) and \( \text{Go}_2 \) \( \alpha \)-subunits. Incubation of membranes of control but not of pertussis toxin-treated cells with \([^{32}]\text{P}\)NAD
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and pertussis toxin led to \[^{32}P\]ADP-ribosylation of three proteins comigrating with the photolabeled and immunologically identified G-protein \(\alpha\)-subunits (Fig. 4C; see also Fig. 8, "Experimental Procedures"). The data show that the photolabeled proteins represent \(\alpha\)-subunits of pertussis toxin-sensitive G-proteins and that pertussis toxin treatment of RINm5F cells (10 ng/ml pertussis toxin for 24 h) resulted in a qualitative modification of G, and \(\alpha\)-subunits.

For identification of \(\alpha_\text{2}-\)adrenoceptor-activated G-proteins in RINm5F cells, we studied the effects of adrenaline and clonidine on photolabeling of membrane proteins. The experiments were performed in the presence of GDP which increases the portion of photolabeling sensitive to receptor agonists in other cell types (30, 31). Under this condition, the proteins comigrating with the \(G_\alpha\)-\(\alpha\)-subunits and the \(G_{12}\) \(\alpha\)-subunit were photolabeled to comparable extents (Fig. 5); relatively little radioactivity was incorporated in the protein comigrating with the \(G_1\) \(\alpha\)-subunit. Adrenaline and clonidine dose-dependently stimulated photolabeling of the proteins comigrating with the two \(G_\alpha\)-\(\alpha\)-subunits and the \(G_{12}\) \(\alpha\)-subunit. An effect on photolabeling of the protein comigrating with the \(G_1\) \(\alpha\)-subunit was not detectable. The effects were observed at agonist concentrations \(\geq 0.3 \mu M\); maximal effects were observed at agonist concentrations \(\geq 30 \mu M\). The stimulatory effects of adrenaline (Fig. 6) and clonidine (not shown) were largely reduced by the \(\alpha_2\)-adrenoceptor antagonist, yohimbine, and abolished by the nonselective \(\alpha\)-adrenoceptor antagonist, phentolamine. Prazosin, an \(\alpha_1\)-adrenoceptor antagonist, did not affect agonist-stimulated photolabeling. Thus the effect of adrenaline on photolabeling involves \(\alpha_2\)-adrenoceptors. In membranes of pertussis toxin-treated cells, photolabeling of proteins was reduced indicating a reduced basal activity of G-proteins (see also Fig. 3). Pertussis toxin abolished the stimulatory effect of adrenaline (see Fig. 6) and clonidine (not shown) on photolabeling. The data indicate that activated \(\alpha_2\)-adrenoceptors interact with two \(G_\alpha\)-\(\alpha\)-subunits and the \(G_{12}\) \(\alpha\)-subunit.

Somatostatin and galanin also enhanced photolabeling of G-protein \(\alpha\)-subunits (data not shown). The effect of either peptide employed at a concentration of 1 \(\mu M\) was considerably less than that of adrenaline employed at maximally effective concentrations. The effect of somatostatin (up to 1 \(\mu M\)) was qualitatively similar to that of adrenaline. Galanin (up to 1 \(\mu M\)) stimulated photolabeling of the proteins comigrating with the putative \(G_\text{Gic}\), \(\alpha\)-subunit and the \(G_\alpha\)-\(\alpha\)-subunit; an effect on photolabeling of the protein comigrating with the \(G_{12}\) \(\alpha\)-subunit was not observed. Employed at very high concentrations (>1 \(\mu M\)), galanin and somatostatin also stimulated photolabeling of the protein comigrating with the \(G_1\) \(\alpha\)-subunit.

**DISCUSSION**

In this report we show that adrenaline inhibits Ca\(^{2+}\) currents in rat insulinoma cells (RINm5F) by about 50%. The effect of the hormone requires activation of \(\alpha_2\)-adrenoceptors, is mediated by pertussis toxin-sensitive G-proteins, and does not involve a cAMP-dependent intermediate step. Other inhibitors of insulin secretion, somatostatin and galanin, also reduce Ca\(^{2+}\) currents by 20 and 38%, respectively. Whereas the effect of the former peptide is abolished by pretreatment of cells with pertussis toxin, the effect of the latter is not influenced by the toxin. Thus, the Ca\(^{2+}\) current inhibition induced by galanin does not require activation of receptors coupled to pertussis toxin-sensitive G-proteins. In contrast, the galanin-induced stimulation of ATP-dependent K\(^+\) channels is sensitive to pertussis toxin (11, 12). Therefore, galanin may inhibit Ca\(^{2+}\) currents by acting on a signal-transduction-component distal of the receptor, e.g. the channel protein or an unknown regulatory component. Alternatively, galanin receptors may additionally couple to G-proteins which are not substrates for pertussis toxin. The present data indicate that Ca\(^{2+}\) current inhibition is a general feature of inhibitors of insulin secretion. Since somatostatin and galanin but not adrenaline have been shown to stimulate ATP-dependent K\(^+\) channels (11, 12), the two peptides may inhibit insulin secretion by a more complex mechanism than does adrenaline. During the course of our studies, adrenaline and somatostatin-induced inhibitions of Ca\(^{2+}\) currents in HIT cells were reported (32, 33).

Pertussis toxin-sensitive inhibition of voltage-dependent Ca\(^{2+}\) currents by receptor agonists including adrenaline, dopamine, \(\gamma\)-aminobutyric acid (via GABA\(_B\) receptors), opioid peptides, neuropeptide Y, and acetycholine (via muscarinic receptors) has been observed in a variety of neuronal cells (16, 17). Recent data indicate that the inhibitory modulation of neuronal Ca\(^{2+}\) currents may be causally related to the inhibition of neurosecretion via presynaptic receptors (18). In pituitary cells, the pertussis toxin-sensitive inhibition of voltage-dependent Ca\(^{2+}\) currents (21, 34, 35) may represent a mechanism by which somatostatin and acetycholine inhibit hormone secretion. By analogy, the inhibition of Ca\(^{2+}\) currents by adrenaline, somatostatin, and galanin in insulin-secreting cells may represent a mechanism for the inhibitory control of insulin secretion. Since adrenaline, somatostatin, and galanin maintain their ability to inhibit insulin secretion in permeabilized cells in a pertussis toxin-sensitive manner (13–15), other mechanisms which are independent of transmembrane ion fluxes and the cytosolic Ca\(^{2+}\) concentration are likely to exist.

We found that the by far major portion of Ca\(^{2+}\) currents in RINm5F cells was sensitive to dihydropyridines. The dihydropyridine isradipine (1 \(\mu M\)) reversibly reduced Ca\(^{2+}\) currents by about 80%. Findlay and Dunne (36) reported that inhibition of Ca\(^{2+}\) currents in RINm5F cells by 85% required the dihydropyridine nifedipine at a very high concentration (50 \(\mu M\)). This discrepancy in the sensitivity of RINm5F cell Ca\(^{2+}\) currents towards dihydropyridines is possibly due to the difference in potency of the two compounds and to the fact that Findlay and Dunne used a lower stimulation frequency (0.2 Hz) than we did (0.5 Hz). Since adrenaline inhibited Ca\(^{2+}\) currents by 50%, the hormone-sensitive currents represent at least in part currents through dihydropyridine-sensitive Ca\(^{2+}\) channels. Adrenaline apparently reduced the initial rate of current inactivation; therefore, an inhibitory effect of the hormone on a fast inactivating current component cannot be excluded. In HIT cells, adrenaline reduces rapidly and slowly decaying Ca\(^{2+}\) currents, both of which are sensitive to the dihydropyridine, nimodipine (4, 32).

We also employed the snail toxin, \(\omega\)-conotoxin, to characterize Ca\(^{2+}\) currents of RINm5F cells. According to recent reports (23, 24), the toxin specifically inhibits Ca\(^{2+}\) currents through N-type channels in neuronal cells. \(\omega\)-Conotoxin had virtually no effect on Ca\(^{2+}\) currents in RINm5F cells and did not modify the Ca\(^{2+}\) current inhibition induced by adrenaline. In neuronal cells catecholamines and other receptor agonists inhibit voltage-dependent Ca\(^{2+}\) currents which are sensitive to \(\omega\)-conotoxin and are, therefore, assumed to represent currents through N-type channels (17, 18, 22). Thus, depending on the cell type, pertussis toxin-sensitive G-proteins may be involved in the receptor-mediated inhibition of various types of Ca\(^{2+}\) channels. Evidence for the involvement of pertussis toxin-sensitive G-proteins in the hormonal inhibition of dihy-
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dropping-pyridine-sensitive Ca\(^{2+}\) channels is also suggested by data obtained with pheochromocytoma (PC12) cells (37). The inhibitory action of adrenaline requires activation of \(\alpha_2\)-adrenoceptors and is mediated by pertussis toxin-sensitive G-proteins. \(\alpha_2\)-Adrenoceptors are assumed to reduce cAMP levels by inhibition of adenylcyclase via a G-protein of the G\(_2\) family, possibly G\(_{12}\) (38). Loading of cells with cAMP did not influence the adrenaline-induced inhibition of Ca\(^{2+}\) currents. This observation is analogous to the cAMP-independent inhibition of Ca\(^{2+}\) currents in neuronal, pituitary, and pheochromocytoma (PC12) cells by receptor agonists known to induce inhibition of adenylcyclase (16, 17, 37). Since cAMP is the only known cytosolic signal molecule controlled via \(\alpha_2\)-adrenoceptors and pertussis toxin-sensitive G-proteins (39) and apparently not causally related to Ca\(^{2+}\) current inhibition, \(\alpha_2\)-adrenoceptors may induce inhibition of Ca\(^{2+}\) currents by a membrane-confined mechanism, as has been suggested for the inhibitory Ca\(^{2+}\) current modulation by receptor agonists in neuronal cells (16, 17). The present experimental conditions also exclude the possibility that Ca\(^{2+}\) current inhibition in RINm5F cells is secondary to stimulation of hyperpolarizing K\(^+\) currents via \(\alpha_2\)-adrenoceptors (40).

A detailed analysis of G-proteins in insulin-secreting pancreatic B-cells is lacking. Therefore, membranes of RINm5F cells were probed with various sequence-specific antibodies directed against G-protein \(\alpha\)-subunits. We identified two G\(_{12}\) \(\alpha\)-subunits which may correspond to the two forms found in brain and other tissues (7, 41). The existence of G\(_{12}\) \(\alpha\)-subunits in insulin-secreting cells is consistent with immunohistochemical studies performed by Asano and coworkers (42), who found G\(_{12}\), \(\alpha\)-subunits in the islets of Langerhans but not in the exocrine pancreas. By screening a HIT cell cDNA library, Hsu and coworkers (42) recently isolated two G\(_{12}\), \(\alpha\)-subunit-encoding cDNAs, \(\alpha_{12}\) and \(\alpha_{22}\), which apparently are derived from a single transcript by alternative splicing. Whereas \(\alpha_{12}\) mRNA was detected in RINm5F cells the evidence for the existence of \(\alpha_{22}\) mRNA was not conclusive (28). Our data show that RINm5F cells express the G\(_{12}\), \(\alpha\)-subunit. Since \(\alpha_{12}\) mRNA is present in RINm5F cells, and since only two translation products of the G\(_{12}\), \(\alpha\)-subunits are known (27), the immunologically not classified G\(_{12}\), \(\alpha\)-subunit is likely to correspond to the G\(_{12}\), \(\alpha\)-subunit.

In addition, two forms of the G\(_{12}\), \(\alpha\)-subunit were detected in membranes of RINm5F cells, which were further identified as \(\alpha\)-subunits of G\(_{12}\) and G\(_{2}\) by the use of subtype-specific antibodies. An \(\alpha_2\) peptide antibody which detected G\(_{12}\), \(\alpha\)-subunits in membranes of HL-60 cells known to possess G\(_{12}\) (43) failed to recognize a protein in the 40-kDa region in membranes of RINm5F cells. Thus, G\(_{12}\) is either not expressed in RINm5F cells or expressed at very low levels. The pattern of pertussis toxin-sensitive G-proteins in RINm5F cells resembles that of brain tissue, which contains G\(_{10}\), G\(_{12}\), G\(_{11}\), and G\(_{2}\), whereas G\(_{3}\) is absent or occurs at low levels (41, 43, 44). An exception are neuroblastoma × glioma hybrid cells (NG 108-15) which reportedly express readily detectable levels of G\(_{2}\), but essentially undetectable levels of G\(_{3}\) (45). Nonneuronal tissues and cells typically possess G\(_{2}\) and G\(_{3}\) but not G\(_{12}\) (43). With the exception of pituitary and neuroendocrine cells, adult nonneuronal cells do not express G\(_{12}\) (42).

In order to identify G-proteins activated via \(\alpha_2\)-adrenoceptors, we photolabeled membrane proteins with \([\alpha-\text{32P}]\text{GTP azidoanilide}\). In contrast to reconstitution studies with purified G-proteins, this approach enabled us to study "in situ" the interaction of activated receptors with endogenous G-proteins. Adrenaline and clonidine stimulated incorporation of \([\alpha-\text{32P}]\text{GTP azidoanilide}\) into three pertussis toxin sub-units, comigrating with the two forms of the G\(_{12}\), \(\alpha\)-subunits and the G\(_{2}\), \(\alpha\)-subunit. A stimulatory effect was not observed in membranes of pertussis toxin-treated cells. Pharmacological characterization showed that the effect was mediated by \(\alpha_2\)-adrenoceptors. Thus, \(\alpha_2\)-adrenoceptors appear to interact with multiple G-proteins which belong to the G\(_2\) and G\(_{12}\) families. A similar observation was made in membranes of neuroblastoma × glioma hybrid cells (31). Our findings are consistent with those reported by Cerione and colleagues, who showed that purified \(\alpha_2\)-adrenoceptors reconstituted into phospholipid vesicles activate purified G\(_{2}\) and G\(_{12}\) (46). The data indicate that ramification of transmembranous signaling occurs on the level of G-proteins; each activated G-protein may specifically affect the activity of an effector, e.g. adenylcyclase and ion channels for K\(^+\) and Ca\(^{2+}\). Alternatively, subtypes of \(\alpha_2\)-adrenoceptors (47) may specifically interact with a single G-protein species each.

It is not clear from the present data which of the G-proteins activated via \(\alpha_2\)-adrenoceptors confers inhibitory effects to voltage-dependent Ca\(^{2+}\) channels of RINm5F cells. Whereas G\(_{12}\) is assumed to confer inhibition from activated receptors to adenylcyclase (39), G\(_{2}\) is neither member of the G\(_2\) family likely to confer inhibition from activated opioid receptors, \(\alpha_2\)-adrenoceptors and receptors for neuropeptide Y to neuronal voltage-dependent Ca\(^{2+}\) channels (see Ref. 17 for review). Only in the case of receptors for bradykinin, there is equally good evidence for the involvement of G\(_{12}\) and G\(_{2}\) in the inhibition of Ca\(^{2+}\) channels in dorsal root ganglion neurons (48). Our data indicate that \(\alpha_2\)-adrenoceptors in membranes of RINm5F cells activate two forms of G\(_{2}\). Therefore, G\(_{2}\) may functionally couple \(\alpha_2\)-adrenoceptors and voltage-dependent Ca\(^{2+}\) channels in insulin-secreting cells, too. Future work should clarify whether receptor- and G\(_{2}\)-mediated inhibition of voltage dependent Ca\(^{2+}\) channels provides a molecular mechanism for the inhibition of secretion as a specific feature of neuronal, pituitary, and neuroendocrine cells.

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REFERENCES

Inhibition of Ca2+ Currents in Insulin-Secreting Cells

SUPPLEMENTARY MATERIAL TO
INVESTIGATION OF PYRROOLIDINE-SENSITIVE Ca2+ PROTEINS IN THE HOMOLOGOUS INHIBITION OF GLUCOSE/AMINO ACID-SENSITIVE Ca2+ CURRENTS IN AN IN-VITRO INSULIN-SECRETING CELL LINE (RINm5F)

by


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Experimental Procedures

Culture of RINm5F cells. Rat islets of Langerhans (RINm5F) were cultured as monolayers in RPMI 1640 medium containing 10% fetal calf serum (FCS), 25 mM HEPES, 250 μg/ml streptomycin, 250 U/ml penicillin, and 50 μg/ml gentamicin in a 37°C, 5% CO2 incubator. After 2-3 days, the cells were harvested with trypsin, washed with phosphate-buffered saline (PBS), and resuspended in the appropriate medium for experiments. For electrophysiological experiments, cells were cultured on coverslips that were coated with a 0.1% gelatin/0.1% collagen/0.05% polylysine solution.

Determination of Ca2+ (Ca++) currents. For electrophysiological studies, cells were seeded at a density of 20-50 cells/cm2 in a 6-well plate (diameter 3.6 cm) and grown in 5% CO2 in the incubator. After 3-5 days, the cells were washed with PBS and incubated in Tyrode solution containing 10 mM HEPES, 115 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 1 mM MgCl2, 5 mM glucose, and 0.1% BSA. The Tyrode solution was then replaced with a solution containing 10 mM HEPES, 115 mM NaCl, 5 mM KCl, 0.5 mM EGTA, and 20 mM HEPES (pH 7.4). After 3 min of preincubation, samples were incubated for 3 min with 5 mM [Ca++]j and 5 μM of a [Ca++]j indicator dye (Fluo-3 AM). The Ca2+ influx was measured by fluorescence microscopy using a confocal microscope. The images were acquired using a 63× objective and a 488 nm excitation wavelength. The fluorescence intensity was measured using a fluorescence multiplier (Fluoromax-3, Jobin Yvon) and analyzed using the Signal software (Jobin Yvon).

Culture and Electrophysiological Studies. The cells were cultured on glass coverslips for electrophysiological recording. The glass coverslips were coated with 0.1% gelatin/0.1% collagen/0.05% polylysine and 1% BSA. After 2-3 days, the cells were washed with PBS and incubated in Tyrode solution containing 10 mM HEPES, 115 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 1 mM MgCl2, 5 mM glucose, and 0.1% BSA. The Tyrode solution was then replaced with a solution containing 10 mM HEPES, 115 mM NaCl, 5 mM KCl, 0.5 mM EGTA, and 20 mM HEPES (pH 7.4). After 3 min of preincubation, samples were incubated for 3 min with 5 mM [Ca++]j and 5 μM of a [Ca++]j indicator dye (Fluo-3 AM). The Ca2+ influx was measured by fluorescence microscopy using a confocal microscope. The images were acquired using a 63× objective and a 488 nm excitation wavelength. The fluorescence intensity was measured using a fluorescence multiplier (Fluoromax-3, Jobin Yvon) and analyzed using the Signal software (Jobin Yvon).

Determination of Ca2+ Currents in Insulin-Secreting Cells. The Ca2+ currents were determined in the whole-cell configuration or the perforated patch configuration. Ca2+ influx was measured by recording the change in cell capacitance. The capacitance change was calculated using the following equation:

\[ \Delta C = \frac{Q}{V} \]

where ΔC is the capacitance change, Q is the charge, and V is the voltage.

The capacitance change was then converted to the Ca2+ influx using the following equation:

\[ \text{Ca2+ influx} = \frac{\Delta C \times V}{2.303 \times R \times F} \]

where ΔC is the capacitance change, V is the voltage, R is the specific resistance of the cell membrane, and F is the Faraday constant.

Electrophysiological recordings were performed in the whole-cell configuration using a patch-clamp amplifier (Axopatch 200B, Axon Instruments). The cells were held at a constant voltage of -60 mV, and the currents were recorded using a low-capacitance patch pipette (3-5 MΩ). The recording solution contained (in mM): 140 NaCl, 2 KCl, 1 CaCl2, 1 MgCl2, 10 glucose, 10 HEPES, and 0.1% BSA. The pH was adjusted to 7.4 using NaOH.

The voltage clamp was used to clamp the cell membrane to a holding potential of -60 mV. The cells were then stimulated with 10 mM KCl, and the Ca2+ currents were recorded using a low-capacitance patch pipette (3-5 MΩ). The current records were filtered at 5 kHz, digitized at 20 kHz, and digitized with a 16-bit A/D converter (Digidata 1440A, Axon Instruments). The data were acquired and analyzed using pClamp 10.7 software (Axon Instruments). The Ca2+ currents were measured using the pClamp software (Axon Instruments).

Fig. 7. Characterization of antibodies raised against peptides specific for G18, a subunit. Fusion proteins of G18 and G18c were expressed in Escherichia coli with a 17 promoter-based expression system (56, 57). Using 488 nm of bacterial lysate (obtained with sample buffer according to Laemmli, ref. 51), the monoclonal antibody (Pharmingen) recognized comparable signals within the Ca2+ and G18c subunit fusion proteins. The proteins of the monoclonal antibody were identified using the log-emission spectrum. For inhibition experiments, the Ca2+ currents were measured using the pClamp software (Axon Instruments).
Inhibition of Ca^{2+} Currents in Insulin-Secreting Cells

Fig. 8:  α-subunits in membranes of RINm5F cells. Panels A and B: Membranes of control cells (panel A) or pertussis toxin-treated cells (panel B) were photolabeled with [32P]ADP-ribosylated factor (SOS-ADP-ribosylase) in the absence of GDP. The 32P concentration was 5 μM. Further, membranes were applied half of the width of a 4 M urea-containing SDS gel (panel A: 100 μg; panel B: 165 μg). Proteins were separated and transferred onto nitrocellulose filters. The filters were cut into strips which were autoradiographed (left and right lanes of panels A and B) and subsequently immunostained with the indicated antibody (5 central lanes): α2, α2 peptide antibody; α2, α4 common peptide antibody; d2, d2 peptide antibody; α2, α4 peptide antibody. Figures on the left margin indicate the molecular masses of marker proteins. In contrast to the preparation of the α4 common peptide antibody used in the experiment shown in Fig. 4, the preparation used in this experiment recognized additional α-subunit. Panel C: Membranes of RINm5F cells, the α2 peptide antibody recognized a 43 kDa protein substrate (α2 α-subunit) and an additional protein in the 40 kDa region which did not shift upon treatment of cells with pertussis toxin. Due to the pertussis toxin-induced shift of the 43 kDa pertussis toxin substrate, the other immunoreactive protein became "unmasked" in membranes of pertussis toxin-treated cells (compare Figs. A and B). The identity of this protein is unknown. Panel C: Membranes (50 μg protein per assay tube) of control cells (left lane) or pertussis toxin-treated cells (right lane) were 32P-ADP-ribosylated (32P-ADPR) by incubation with [32P]NAD (4). Proteins were separated by SDS-polyacrylamide gel electrophoresis in the presence of 4 M urea and blotted onto nitrocellulose filter. Shown is the autoradiogram of the filter.

Fig. 9:  α-subunits in membranes of myeloid differentiated HL-60 and RINm5F cells. Proteins were separated by SDS PAGE in the presence of 4 M urea. Shown are nitrocellulose filters which were immunostained with the indicated antibody: α2, α2 common peptide antibody; α2, α2 peptide (1 antibody); α2, α2 peptide antibody; α2, α2 peptide antibody. The amounts of protein applied per lane were 50 μg (IC.1, 2, 2) or 100 μg (J3). The preparation of the α2 common peptide antibody used in this experiment recognized in membranes of RINm5F cells an additional protein of 45 kDa. Other preparations of this antibody did not react with the 45 kDa protein (see Fig. 4). It is therefore unlikely to correspond to α2 α-subunit. For culture of HL-60 cells and preparation of membranes see ref. 30.

Fig. 10:  [32P] ATP hydrolysis in membranes of RINm5F cells (isotope dilution experiment). Per assay tube, 6 μg of membrane protein were employed. The ATP concentration is indicated on the abscissa, the percentage of hydrolyzed [32P] ATP on the ordinate. Values are mean values of triplicates with an intrasay variation of less than 5% of total ATPase activity.

Fig. 11:  High-affinity ATPase activity in membranes of RINm5F cells as a function of time and protein content per assay tube. Values are mean values ± SD (n = 4). The incubation time is indicated on the abscissa, the protein content per assay tube in the figure.